Transport activity of AE3 chloride/bicarbonate anion-exchange proteins and their regulation by intracellular pH

Deborah STERLING and Joseph R. CASEY¹

Department of Physiology, Membrane Transport Group and MRC Group in Molecular Biology of Membranes, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Plasma membrane $\text{Cl}^-/\text{HCO}_3^-$ anion-exchange (AE) proteins contribute to regulation of intracellular pH (pH_i). We characterized the transport activity and regulation by pH_i of full-length AE3 and the cardiac isoform, AE3c, both of which are expressed in the heart. AE3c is an N-terminal variant of AE3. We also characterized AE1, AE2 and a deletion construct (AE3tr) coding for the common region of AE3 and AE3c. AE proteins were expressed by transient transfection of HEK-293 cells, and transport activity was monitored by following changes of intracellular pH or intracellular chloride concentration associated with anion exchange. Transport activities, measured as proton flux ($\text{mM H}^+ \cdot \text{min}^{-1}$), were as follows: AE1, 24; AE2, 32; full-length AE3,

9; AE3c, 4 and AE3tr, 4. The wide range of transport activities is not explained by variation of cell surface processing since approx. 30 % of each isoform was expressed on the cell surface. pH_i was clamped at a range of values from 6.0–9.0 to examine regulation of AE proteins by pH_i . Whereas AE2 was steeply inhibited by acid pH_i , AE1, AE3 and AE3c were essentially insensitive to changes of pH_i . We conclude that AE3 and AE3c can contribute to pH_i recovery after cellular-acid loading.

Key words: anion exchange, cardiomyocytes, cell acidosis, ischaemia, reperfusion.

INTRODUCTION

Anion-exchanger (AE) proteins facilitate the electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane of mammalian cells and thus contribute to regulation of intracellular pH (pH_i), $[\text{Cl}^-]$ and cell volume. The erythroid band 3 protein, also called AE1, was the first anion exchanger identified [1]. cDNA libraries from a range of other tissues were probed with the AE1 gene, leading to the identification of other members of the AE family. The anion exchanger family is now comprised of three members, AE1, AE2 and AE3 [2,3].

The AE3 isoform is of particular interest because it is found in the excitable tissues, brain [2], heart [4] and retina [5]. Heart and retina co-express two different isoforms of AE3, which result from alternative promoter usage [4–6]. The 5'-untranslated and the 5'-coding sequences of the full-length mRNA are contained within exons 1–6 and the corresponding sequences of the cardiac isoform of AE3c (AE3c) mRNA are contained within intron 6 [4]. Exons 7–23 encode sequences common to both full-length AE3 and AE3c [4]. Human full-length AE3 is 1232 amino acids long and AE3c contains 1034 amino acids, including the unique 73-amino-acid domain at the N-terminus (Figure 1) [7]. AE3c is the most abundant AE protein expressed in the heart [6], whereas in the retina AE3c and full-length AE3 are expressed at similar levels, but in different retinal cell layers [5].

pH_i modulates diverse cellular events, including ion-channel conductance [8], Ca^{2+} homeostasis [9], gene expression [10] and cell death [11]. In cardiac muscle, Ca^{2+} homeostasis and Ca^{2+} sensitivity of myofilaments, which closely regulate contraction, are affected by changes of pH_i [12]. pH_i also modulates gap-

junction conductance and, in turn, cardiac-cell electrical coupling [8,13].

During a cardiac ischaemic episode, heart cells develop an acid load from which they must recover. Intracellular buffers and ion-transport proteins present in the plasma membrane mediate regulation of pH_i in cardiac cells. An understanding of the function and regulation of pH-regulatory proteins would help to

Figure 1 Structural homology of AE3 isoforms

The C-terminal 957 amino acids of AE3 isoforms are identical, but the cardiac isoform has a unique 73 amino acid domain at its N-terminus (solid bar). AE3tr contains only the region in common between full-length AE3 and AE3c. The striped portions represent the transmembrane domain. Arrows indicate epitopes recognized by antibodies.

Abbreviations used: AE, anion exchanger; AE3, AE protein 3; AE3c, cardiac isoform of AE3; AE3tr, truncated isoform of AE3; AP3, a polyclonal antibody directed against the common C-terminus of all AE3 variants; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester; cAE3-1, a polyclonal antibody directed against the unique region of AE3c; CA, carbonic anhydrase; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; pH_i , intracellular pH; pH_o , extracellular pH; SPQ, 6-methoxy-N-(3-sulphopropyl)quinolinium.

¹ To whom correspondence should be addressed (e-mail joe.casey@ualberta.ca).

elucidate their role in the recovery of pH_i in cardiomyocytes. Membrane-ion-transport proteins present in cardiomyocytes include the Na^+/H^+ antiporter [14], the $\text{Na}^+/\text{HCO}_3^-$ co-transporter [15], the lactate/ H^+ co-transport proteins [16] and isoforms of the AE family [6,17]. In addition to the two AE3 variants, cardiac cells also express AE2, at least at the mRNA level [18]. Full length AE1 has been detected in the heart [18], and a truncated form of AE1 has also been reported [17].

The anion-exchange activity of AE1 and AE2, and the pH_i -sensitivity of transport have been studied previously [19–21]. AE1 transport activity is known to be relatively insensitive to changes in pH_i over a physiological range [19]. AE2, however, is under steep negative regulation at acid pH_i , consistent with its role in recovery from alkaline loading [20,21]. The regulation of full-length AE3 by pH_i has been reported previously [22], but regulation of AE3c has not been studied.

In the present study we used transiently transfected HEK-293 cells to examine the anion-transport activity of both the full-length and cardiac isoforms of AE3 that are found in the heart. We then determined the role of pH in regulation of AE activity. We found that AE3 and AE3c are not sharply regulated by pH. The transport activity of AE3 isoforms at acid pH suggests that these proteins may be involved in recovery from acid loading, by inward transport of HCO_3^- .

EXPERIMENTAL

Materials

ECL[®] enhanced chemiluminescence reagent, donkey anti-rabbit IgG conjugated to horseradish peroxidase and Hyperfilm were from Amersham–Pharmacia Biotech. Poly-L-lysine and nigericin were from Sigma–Aldrich Canada. 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR, U.S.A.) and 6-methoxy-*N*-(3-sulphopropyl)quinolinium (SPQ) was from Toronto Research Chemicals (Toronto, Ontario, Canada). Glass coverslips were from Fisher Scientific Products. Sulpho-NHS-SS-Biotin and streptavidin–agarose were from Pierce Chemical Company (Rockford, IL, U.S.A.). Sheep anti-(human carbonic anhydrase II) antibody was from Serotec (Raleigh, NC, U.S.A.). Donkey anti-(sheep IgG) conjugated to horseradish peroxidase was from BioCan Scientific (Mississauga, Ontario, Canada).

Molecular biology

cDNA clones for rat full-length AE3 and AE3c were kindly provided by Dr. Gary Schull [4,18]. The *Xba*I/*Eco*RI fragment of the *AE3c* cDNA was cloned into those sites in the expression vector pRBG4 [22] to yield construct pJRC31. An expression construct for full-length AE3 was prepared by replacement of the 5' end of the pJRC31 coding region. PCR was performed using the rat *AE3* cDNA clone pRBAE2-1 as template with forward primer A6257G07 (CGG AAT TCT CTA GAT GAG ACT CCT AAG TCT TCA GCC) and reverse primer A6257G09 (TCC GAT CCA GCT TCT TCT TC). The resulting PCR product was digested with *Xba*I and *Nar*I and cloned into pJRC31, digested with the same enzymes, to produce construct pJRC32. A deletion construct encoding the common region found in AE3 and AE3c (designated AE3tr) was designed to contain an additional ATG start codon at the 5' end. The truncated construct, called pJRC34, was prepared by a deletion PCR strategy [23]. In the first round of PCR, pJRC31 was the template for PCR with forward primer A7723F07 (CTG AGG CCT TGG CCA CCA GGG GAA TGT GTC ATC GGC TGG AGG ACA ACC CG) and reverse primer A6257G09. This

megaprimer was isolated and used as a reverse primer with forward primer A7728E03 (GCC ATC CAC GCT GTT TTG ACC), which has sequence similarity to the multiple cloning site of pRBG4. The resulting PCR product was digested with *Xba*I and *Nar*I and cloned into pJRC31 digested in the same way.

An expression construct of glutathione S-transferase cDNA fused to codons 7–69 of rat *AE3c* was constructed by PCR with rat *AE3c* cDNA (from Dr. Gary Schull) as template, forward primer 2693 (CCG GGA TCC ACA AGG TCA TGG AGC CC) and reverse primer 2692 (CCG GGA TCC GCT CCC ACA GGC CAT AGT C), which introduced a *Bam*HI site at each end of the coding sequence for the unique domain of *AE3c*. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pGEX-5X-3 (Amersham-Pharmacia Biotech) to yield pJRC10.

A construct for expression of human carbonic anhydrase II (CAII) was prepared by digestion of pACA (containing the CAII cDNA) with *Xba*I and *Eco*RI. The pACA plasmid was a gift from Dr. Carol Fierke [24]. The *Xba*I–*Eco*RI fragment of pACA was cloned into *Xba*I and *Eco*RI digested pRBG4 vector, to yield pJRC36.

All constructs were verified by DNA sequencing, performed by the Core Facility in the Department of Biochemistry, University of Alberta, with an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Plasmid DNA for transfections was prepared using Qiagen columns.

Protein expression

AEs were expressed by transient transfection of HEK-293 cells [25] using the calcium phosphate method [26]. Cells were grown at 37 °C in an air/ CO_2 (19:1) environment in Dulbecco's modified Eagle's medium ('DMEM') (Canadian Life Technologies Inc., Burlington, Ontario, Canada), supplemented with 5% (v/v) foetal bovine serum (Canadian Life Technologies Inc.) and 5% (v/v) calf serum (Canadian Life Technologies Inc.). Cells were used two days post transfection.

Antibody preparation

Construct pJRC10 was transformed into Top 10-F' *Escherichia coli*. Fusion protein was expressed and purified, following directions from the manufacturer of the pGEX-5X-3 vector. Two rabbits were each immunized subcutaneously with 800 μg of fusion protein in Freund's complete adjuvant. Rabbits were then immunized twice at monthly intervals subcutaneously with 500 μg of fusion protein in Freund's incomplete adjuvant. Rabbits were killed, exsanguinated and crude sera designated cAE3-1 were isolated.

Immunoblot analysis

HEK-293 cells, grown in 60 mm (diameter) tissue culture dishes, were transiently transfected with pJRC31, pJRC32 or pJRC34 plasmids to induce expression of the AE3c, full-length AE3 or AE3tr isoform respectively, as described above. Two days post transfection, cells were washed in PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) and lysates of the whole tissue culture cells were prepared by addition of 250 μl of SDS/PAGE sample buffer [10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.5% (w/v) bromophenol blue, 75 mM Tris, pH 6.8] containing, 0.1 mM PMSF, 0.2 mM tosyl-phenylalanylchloromethane ('TPCK'), 0.1 mM tosyl-lysyl-chloromethane ('TLCK') and 2 mM EDTA. Samples were stored on ice while being sheared through a 26 gauge needle

(Becton–Dickinson). Prior to analysis, samples were heated to 65 °C for 5 min and insoluble material was sedimented by centrifugation at 16000 *g* for 10 min in an IEC Micromax microcentrifuge. Samples were resolved by SDS/PAGE on 8% (w/v) acrylamide gels [27]. Proteins were transferred to PVDF membranes by electrophoresis for 12 h at 30 V in a cold room, in buffer composed of 20% (v/v) methanol, 25 mM Tris and 192 mM glycine. PVDF membranes were blocked by incubation for 1 h in TBST-M {TBST [0.1% (v/v) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5], containing 5% (w/v) non-fat dry milk} and then incubated overnight with 10 ml of TBST-M containing either 1 μ l Serotec anti-CAII antibody, 3 μ l 1658 rabbit anti-AE1 polyclonal antibody [28], 3 μ l rabbit anti-AE3 antibody (AP3) [5] or 2 μ l rabbit anti-AE3c antibody (cAE3-1). After washing with TBST, blots were incubated for 1 h with 10 ml of TBST-M containing 1:3000 diluted donkey anti-(rabbit IgG) conjugated to horseradish peroxidase. Anti-CAII immunoblots were incubated with 1:10000 diluted donkey anti-(sheep IgG) conjugated to horseradish peroxidase. After washing with TBST, blots were visualized using ECL[®] reagent and Hyperfilm as previously described [28].

Anion-exchange-activity assay

HEK-293 cells, grown on poly-L-lysine-coated coverslips in 60 mm dishes, were transfected with pJRC31, pJRC32, or pJRC34 as described previously [26]. Mouse AE2 and human AE1 were expressed by transfection with pRBG4-based plasmids pBSL103 [2] and pJRC9 [29], respectively. Two days post transfection, coverslips were rinsed in serum-free medium and incubated in 4 ml of serum-free medium containing 2 μ M BCECF-AM (37 °C, 30 min). Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO₄, 2.5 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM Hepes pH 7.4) containing either 140 mM NaCl or 140 mM sodium gluconate. Both buffers were continuously bubbled with air/CO₂ (19:1). Fluorescence changes were monitored in a Photon Technologies International (London, Ontario, Canada) RCR fluorimeter at excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. Fluorescence data were converted to pH_i by calibration using the nigericin/high potassium method [30], with pH values of 6.5, 7.0 and 7.5. pH_i values were converted to [H⁺] and transport rates were determined by linear regression of the initial H⁺ equivalent flux, using Kaleidagraph software (Synergy Software, Reading, PA, U.S.A.). In some assays the anion-exchange inhibitor 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS, 100 μ M in gluconate-Ringer's buffer), was perfused through the cuvette for 10 min after a standard transport assay. Residual transport activity was then assessed after washing the cuvette with gluconate-Ringer's buffer for 300 s, followed by a standard transport assay [28].

Measurement of intrinsic buffering capacity and proton flux

Intracellular-buffering-capacity measurements were made by the ammonium-pulse method [31]. HEK-293 cells grown on coverslips were sham transfected as described previously [26]. Two days post transfection, cells were loaded with BCECF-AM as described above. Coverslips were mounted in a fluorescence cuvette and allowed to equilibrate in Ringer's buffer, without NaHCO₃. Cells were then perfused consecutively for 200 s with Ringer's buffer without NaHCO₃, containing 20, 10, 5, 1 and 0 mM NH₄Cl. [NH₄⁺]_i was calculated from the Henderson–Hasselbalch equation, and the intrinsic buffering capacity (β)

was then calculated as $\Delta[\text{NH}_4^+]_i/\Delta\text{pH}_i$ [31]. The total buffering capacity of the system (β_{total}) was then determined as $\beta_{\text{total}} = \beta_i + \beta_{\text{CO}_2}$, where $\beta_{\text{CO}_2} = 2.3[\text{HCO}_3^-]$ [32]. Total proton flux was calculated as:

$$J_{\text{H}^+} = \beta_{\text{total}} \times \Delta\text{pH}_i \text{ [32].}$$

Surface processing

HEK-293 cells, grown in 60 mm tissue culture dishes, were transiently transfected with AE1, AE2, AE3, AE3c or AE3tr cDNAs as described above. Two days post transfection, cells were washed with PBS and harvested by incubation for 5 min at 37 °C with trypsin at 1 mg/ml in PBS. Cells were washed with 4 °C borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂, 10 mM boric acid, pH 9.0), and then incubated for 30 min at 4 °C in 3 ml of Sulpho-NHS-SS-Biotin at 0.5 mg/ml in borate buffer. After washing three times with cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3), cells were solubilized on ice in 500 μ l of IP buffer [1% (w/v) deoxycholic acid, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/Cl, pH 7.5] containing protease inhibitors (0.1 mM PMSF, 0.2 mM tosyl-phenylalanylchloromethane, 0.1 mM tosyl-lysylchloromethane). The cell lysate was centrifuged for 20 min at 16000 *g* and the supernatant collected. Half of the supernatant was retained for later SDS/PAGE analysis. Immobilized streptavidin resin [50 μ l of 1–3 mg streptavidin/ml settled gel as a 50% (v/v) slurry in PBS containing 2 mM Na₂S₂O₃] was added to the remaining supernatant, which was then incubated overnight at 4 °C with gentle rocking. Samples were centrifuged for 2 min at 8000 *g*, and the supernatant collected and retained. Resin was washed five times with IP buffer and proteins were then eluted from the resin by addition of 250 μ l SDS/PAGE sample buffer and incubation at 65 °C for 5 min. Three samples (total protein, unbound fraction and fraction eluted from resin) were analysed by SDS/PAGE and immunoblotting as described above. Immunoblots were scanned with a Scanjet 4C flatbed scanner (Hewlett–Packard, Palo Alto, CA, U.S.A.), calibrated with a Q-14 grayscale (Kodak, Rochester, NY, U.S.A.). Scanned images were quantified using NIH Image 1.60 software (National Institutes of Health, Bethesda, MD, U.S.A.).

Determination of perfusion interval

To determine the time taken for intracellular pH to equilibrate across the plasma membrane, we monitored pH_i in HEK-293 cells plated onto coverslips, transfected with AE1 cDNA and loaded with BCECF-AM as described above. Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer containing 5 μ M nigericin, and either 140 mM KCl or 140 mM KNO₃. The pH of the Ringer's perfusion buffer was maintained at values 6.0, 6.5, 7.0 and 7.5. Fluorescence changes were monitored in a Photon Technologies International RCR fluorimeter at excitation wavelengths of 440 and 502.5 nm and an emission wavelength of 528.7 nm.

pH dependence assay

HEK-293 cells were plated onto coverslips and transfected as described above. Approx. 24 h post-transfection cells were incubated overnight at 37 °C in 4 ml of serum-supplemented Dulbecco's modified Eagle's medium (see above), containing 10 mM SPQ. Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer containing 5 μ M nigericin, and either 140 mM KCl or 140 mM KNO₃. pH of the Ringer's perfusion buffer was maintained at values 6.0, 7.0, 8.0 and 9.0, thereby clamping pH_i at these values.

At pH 6.0, Hepes in the Ringer's buffer was replaced with 10 mM Mes, and at pH 8.0 and 9.0, Hepes was replaced with 10 mM Tris. Fluorescence was monitored at an excitation wavelength of 350 nm and emission wavelength of 438 nm in a Photon Technologies International RCR fluorimeter. To correct for changes of fluorescence that occurred due to dye leakage from cells and cell loss from the coverslip, total changes in fluorescence were normalized. The maximum fluorescence value observed in each transport cycle was set to 1.0 and the minimum was set to 0. Relative transport rates were determined by linear regression of the initial linear phase of each transport curve, using Kaleidagraph software. DIDS inhibition assays were performed as described above at $\text{pH}_o = \text{pH}_i = 6.0$ (where pH_o is extracellular pH) on cells expressing either full-length AE3 or AE3c and loaded with SPQ.

RESULTS

Transient expression of AE cDNAs

All cDNA clones of interest for this study were inserted in the expression vector pRBG4, which places the insert under the control of the human cytomegalovirus immediate early gene promoter. The HEK-293 cell line was used in these experiments because these cells have very low anion-exchange-transport activity [2]. Immunoblots were probed with either AP3 [5], a polyclonal antibody directed against the common C-terminus of all AE3 variants, or cAE3-1, a polyclonal antibody directed

Figure 2 Immunoblots of AE3 isoforms

Cell lysates were prepared from HEK-293 cells transfected with cDNA encoding: lane 1, full-length AE3; lane 2, AE3c; lane 3, AE3tr and lane 4, pRBG4 vector only. (A) Immunoblot probed with a 1:3000 dilution of the polyclonal anti-(AE3 C-terminus) antibody, AP3. (B) Immunoblot probed with a 1:5000 dilution of polyclonal antibody cAE3-1, directed against the unique N-terminus of AE3c.

Figure 3 Anion-exchange-activity assay

Transiently transfected cells, grown on coverslips, were loaded with BCECF-AM and perfused with Ringer's buffer containing either 140 mM NaCl (open bar) or 140 mM Na gluconate (solid bar) as indicated by the bar at the top of the figure. Excitation wavelengths were 440 nm and 502.5 nm and the emission wavelength was 528.7 nm. pH_i was monitored as outlined in the Experimental section. Cells were transfected with cDNA encoding: (A), full-length AE3; (B), AE3c and (C), AE3tr.

against the unique region of AE3c (Figure 1). Figure 2(A) shows that the three AE3 proteins are expressed to a very similar level. Cells transfected with pRBG4 vector alone had no immunoreactive material (Figure 2A). Figure 2(B) illustrates that the polyclonal antibody cAE3-1 detects AE3c only.

AE activity

The anion-exchange assay measures the rate of exchange of Cl^- for HCO_3^- across the plasma membrane mediated by the AE

Figure 4 Intracellular buffering capacity of sham-transfected HEK-293 cells

Each point represents the mean of four measurements $\pm$ S.E.M.

[33]. In this assay, transfected cells were grown on glass coverslips, loaded with BCECF-AM, a pH sensitive fluorescent dye, and mounted in a fluorescence cuvette. The light beam in the fluorimeter illuminates approximately 1×10^4 cells, so that fluorescence data represent the mean response of a large population of cells. After appropriate calibration, changes of fluorescence indicate changes in pH_i . In Cl^- -free medium, Cl^- leaves the cell in exchange for HCO_3^- entry, which induces cell alkalization (Figure 3) [33]. Figure 3 shows typical anion-exchange data for AE3 isoforms. In these experiments, transport rates were determined from the initial slopes of the curves produced as alkalization occurs. To convert flux in units of change of pH/time to flux of proton equivalents/time, it was essential to determine the intrinsic buffering capacity of transfected HEK-293 cells. Figure 4 shows that the intrinsic buffering capacity of transfected HEK-293 cells was negligible at pH values above 7.2. The flux of proton equivalents in units of $mM \cdot min^{-1}$ was therefore determined from the product of the change of pH/time and $(2.3 \times [HCO_3^-])$.

In anion-exchange assays carbon dioxide diffuses into cells to provide a source of bicarbonate for efflux. HCO_3^- is usually produced by CO_2 hydration, mediated by CA. To determine the importance of CA to the anion-exchange process, we used immunoblots to determine that CAII is endogenously expressed in HEK-293 cells (results not shown). The presence of CAII is essential to observe full anion-exchange activity since treatment of AE1-transfected cells with 100 μM acetazolamide (a CA inhibitor) resulted in nearly complete inhibition of anion transport activity (results not shown). To determine whether the endogenous level of CAII found in HEK-293 cells was sufficient to observe full anion-exchange activity, we cotransfected HEK-293 cells with expression constructs for AE1 and CAII. On the basis of immunoblots, CAII was expressed at levels many times the endogenous level. However, within experimental error the transport activity of AE1-CAII-cotransfected cells could not be distinguished from AE1-transfected cells (results not shown). Therefore cotransfection with CAII was not required to observe full anion-exchange activity; in subsequent experiments cells were not cotransfected with CAII.

Table 1 summarizes the transport activity of AE isoforms. AE1 and AE2 had similar transport activities. This rate is

Table 1 Anion-exchange activity and cell-surface processing

Anion-exchange rates are shown as the mean $\pm$ S.E.M. ($n = 4$) and have been corrected for background activity of cells transfected with pRBG4 vector alone. Cell-surface processing is expressed as a percentage of total AE protein present in the cell, and the S.E.M. is shown ($n = 2$).

AE isoform	Proton flux ($mM \cdot min^{-1}$)	Cell-surface expression (%)
AE1	24 ± 2	31 ± 1
AE2	32 ± 3	32 ± 2
AE3	9 ± 2	30 ± 2
AE3c	4 ± 1	31 ± 1
AE3t	4 ± 1	39 ± 1

Figure 5 Cell-surface processing of AE1

HEK-293 cells transfected with AE1 cDNA were labelled with Sulpho-NHS-SS-biotin. Cells were solubilized in SDS/PAGE sample buffer. Half of the sample was loaded into lane 1 (total protein). The remainder was incubated with streptavidin-agarose, to remove biotinylated proteins. The unbound, supernatant fraction was loaded in lane 3. The beads were incubated at 65 $^{\circ}C$ to cleave the biotin-agarose linkage. The supernatant from this preparation was loaded in lane 2.

approx. 3-fold higher than that of the full-length AE3 isoform, a finding that has been noted previously [22]. Both AE3c and AE3tr had lower transport rates (4.43 ± 0.58 and $3.57 \pm 0.58 mM \cdot min^{-1}$, respectively) than the full-length isoform. Negative-control cells transfected with pRBG4 vector alone displayed a level of $0.81 \pm 0.11 mM \cdot min^{-1}$, which is a background rate of 2.5–22% depending on the AE isoform. The background activity of pRBG4-transfected cells was subtracted from all transport data presented here. All AE isoforms were sensitive to DIDS although to different extents. Following incubation with DIDS, the isoforms had the following residual activities: AE1, $18 \pm 7\%$; AE2, $59 \pm 7\%$; full-length AE3, $38 \pm 1\%$; AE3c, $36 \pm 6\%$ ($n = 2$). Therefore AE2 and AE3 are much less sensitive to inhibition by DIDS than AE1.

Surface processing

The range of transport activities for the AE1, AE2 and AE3 isoforms is striking (Table 1). Because anion-exchange assays only measure the functional activity of protein in the plasma membrane, differences in the apparent transport activity of the AE proteins could result from altered levels of processing to the cell surface. To examine this possibility, we measured the level of cell-surface expression of AEs in cells that had been transfected for the same time period as cells used for transport experiments. Membrane-impermeant Sulpho-NHS-SS-Biotin reacts only with primary amines of proteins in the plasma membrane. We identified the biotinylated cell-surface proteins by their binding to streptavidin resin. Figure 5 shows an example of cell-surface-processing results. The amount of AE1 present in lane 3 (fraction not biotinylated) is similar to the total amount of AE1 present (lane 1). Quantification of the data by densitometry showed that 69% of the protein was retained in an inaccessible intracellular location since it could not be biotinylated. The amount of AE1

Figure 6 Perfusion interval required to change pH_i

HEK-293 cells were grown on glass coverslips and transfected with AE1 cDNA. Two days post-transfection, cells were loaded with BCECF-AM and suspended in a fluorescence cuvette. The cuvette was perfused with high potassium Ringer's buffer at various pH values (see bar at the top of the figure), containing $5 \mu\text{M}$ nigericin and either 140 mM KCl (open bar) or 140 mM KNO_3 (solid bar), as indicated by the bar at the top of the figure. Fluorescence was measured with excitation wavelengths of 440 nm and 502.5 nm and an emission wavelength of 528.7 nm.

present in the biotinylated fraction (lane 2) was much lower than the 31% estimated from the fraction that was labelled. We attribute this to an inability to elute bound AE1 from the resin. Table 1 shows that approximately 30% of total protein present of each isoform of AE is processed to the surface membrane. Therefore differences in cell surface processing do not explain the differences in anion-exchange activity that we observed.

Determination of perfusion interval

Prior to measuring anion-exchange activity at various pH values, it was essential to determine the incubation period required to equilibrate pH_i . In this assay, we used nigericin, a K^+/H^+ ionophore, in the presence of high potassium to equilibrate pH across the plasma membrane [30]. This enabled us to alter pH_i by changing pH_o . To determine the length of time required for pH_i to equilibrate with pH_o , we continuously monitored pH_i in BCECF-AM-loaded cells, while we changed the pH of the extracellular Ringer's medium. Figure 6 shows that pH_i rapidly changed upon change of pH_o and pH_i reached equilibrium within 200 s. Figure 6 also shows that change of medium from chloride-Ringer's to nitrate-Ringer's had no effect on pH_i . The results presented in Figure 6 cover only the pH 6–7.5 region, because the BCECF-AM dye ($pK_a = 6.8$) did not accurately report pH_i beyond this pH range. The pH dependence of BCECF-AM did not affect experiments designed to monitor transport activity in the pH 6–9 range (see below) because SPQ dye was used to follow chloride fluxes.

pH-dependence assay

To follow anion transport at various pH values, we loaded transfected cells with SPQ. Fluorescence of this chloride sensitive dye becomes quenched in the presence of chloride, but not nitrate

Figure 7 Measurement of anion exchange at various pH values

HEK-293 cells, grown on coverslips, were transiently transfected with full-length AE3 cDNA. Cells were loaded with SPQ dye and suspended in a fluorescence cuvette. Cells were perfused with Ringer's buffer at various pH values (see bar at the top of the figure), containing $5 \mu\text{M}$ nigericin and either 140 mM KCl (open bar) or KNO_3 (solid bar), as indicated by the bar at the top of the figure. Fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength of 428 nm. Raw transport data (A) were normalized to the same total change in fluorescence in each transport curve (B).

[34]. We therefore monitored changes of intracellular $[\text{Cl}^-]$, as chloride exchanges for nitrate, a substrate of the AE. In these assays, cells were alternately perfused with chloride- or nitrate-containing Ringer's buffer. As pH levels can only drop as low as 6.2 during an ischaemic episode [12], assays were performed at pH values of 6.0, 7.0, 8.0 and 9.0 to give a full range of AE activity versus pH.

Figure 7 shows data from a representative anion transport experiment, with varied $pH_o = pH_i$. The figure shows AE3-transfected cells undergoing perfusion with chloride- and nitrate-containing Ringer's buffer at pH 7.0, 8.0 and 9.0. The maximum unquenched fluorescence level always decreased after the first curve regardless of the pH values used in the experiment. We attribute the decrease in fluorescence to loss of cells from the coverslip and SPQ leakage from cells. To correct for cell and dye loss, each pH transient was normalized to the same fluorescence range. The correction method was validated by experiments in which transport was measured three consecutive times on the same cell sample, at pH 7.0. After normalization of the fluorescence range, measured transport activity was consistent for all three determinations.

In these experiments, transport rates were determined from the initial slopes of the chloride influx or efflux curves. To ensure that changes of pH_i did not cause irreversible changes to the cell, we

Figure 8 Regulation of anion exchange by pH_i

Anion-exchange rates were measured at various pH_i values. Transport rates are plotted relative to the maximum rate observed for each isoform and error bars represent the S.E.M. ($n = 8$). (A) Open circles ($\circ$), AE1; open squares ($\square$), AE2; open triangles ($\triangle$), full-length AE3; closed circles ($\bullet$), AE3c. AE3tr ($\blacksquare$) activity is shown in (B) and pRBG4 activity ($\blacktriangle$) in (C).

monitored transport activity in cells exposed to the pH sequences (6.0, 7.0), (7.0, 6.0), (7.0, 8.0, 9.0) and (9.0, 8.0, 7.0). Each coverslip was exposed to only one sequence of pH values. In each case pH 7.0 was included in the series to allow for normalization between experiments.

Figure 8 summarizes regulation by pH of each AE isoform. AE1 and AE2 served as controls representing AEs that are known to be relatively pH-insensitive [19] and pH-sensitive [20,21], respectively. Our results are consistent with previous determinations of the pH-dependence of AE1 and AE2 transport activity. Our results show that the anion transport activities of full length AE3, AE3c and AE3tr are relatively pH independent. Figure 8 shows that background chloride flux in cells transfected with pRBG4 vector alone did not vary with pH. The absolute level of the background transport activity could not be assessed in $\text{Cl}^-/\text{NO}_3^-$ exchange assays, but represented only 2.5–22% of total transport when measured in $\text{Cl}^-/\text{HCO}_3^-$ exchange assays (Table 1). Cells expressing either full-length AE3 or AE3c were similarly sensitive to DIDS when pH_i is 6.0 as at physiological pH_i . Anion transport at pH 6.0 can therefore be attributed to the AE and not to other processes that may come into effect at lower pH_i values.

DISCUSSION

In the present study, we examined the transport activity of AE1, AE2 and AE3 isoforms and their regulation by pH. AE1- and AE2-transfected cells had the highest anion-exchange activity

(24 and $32 \text{ mM} \cdot \text{min}^{-1}$, respectively). Full-length AE3 has approximately one third the activity of the AE1- and AE2-transfected cells. It is especially interesting to note that both the truncated AE3 isoforms (AE3c and AE3tr) have considerably lower transport rates than the full-length isoform. This implies that the N-terminus of full length AE3 confers an increase of transport activity on this isoform. All of the AE3 isoforms had anion transport activity that was not inhibited by acid pH_i . This is the first paper to assess the transport activity and regulation by pH of AE1, AE2 and AE3 isoforms in one study. AE3c and the AE3tr mutant have not previously been heterologously expressed in mammalian cells and functionally characterized.

Two previous papers have reported the activity of AE3, after expression in HEK-293 cells [22] and in *Xenopus laevis* oocytes [7] and found different results. Our results are consistent with a report that in HEK-293 cells full-length AE3 activity was 3-fold lower than that of AE2 [22]. Both full-length AE3 and AE3c had similar levels of transport activity, approx. 2.5 times that in water-injected cells, when expressed in *X. laevis* oocytes [7]. By contrast oocytes injected with cRNA for AE1 and AE2 had ion fluxes that were > 10 –20-fold above those in water-injected cells [20]. Thus in *X. laevis* oocytes, AE3 isoforms are about 8-fold less active than AE1 or AE2.

We reasoned that the degree of cell-surface processing of the AE proteins could explain the large differences in their transport activity. Our finding that a similar fraction (approx. 30%) of each AE isoform is processed to the plasma membrane does not explain the differences in transport rates. However, because we do not have an antibody that detects both AE2 and AE3, we cannot rule out the possibility that the transport rates differ because of differences in the level of protein expression. Since the membrane domains, which carry out anion exchange, are 90% identical between AE2 and AE3, the more divergent cytoplasmic domains (53% identity) may account for the differences in transport activity. The cytoplasmic domains of AE proteins may interact with the membrane domain to regulate activity. Indeed, the transport studies of chimaeric proteins of AE1 and AE2 membrane and cytoplasmic domains showed that both domains contribute to regulation of anion transport by pH [35].

Alternatively, tissue-specific cofactors may be required for full function of the AE3 isoforms. In contrast with the HEK-293 cells used in the present study, excitatory cells (heart and retina), where AE3 isoforms are found, may provide the optimal environment for AE3 transport regulation. The reduced anion-exchange activity of AE3tr and AE3c relative to the activity of the full-length AE3 indicates that the unique N-terminal region of the full-length protein contributes to positive regulation of anion-exchange activity. Whether the N-terminal region of AE3 increases transport by interaction with modulatory proteins or regulatory phosphorylation are questions that await further study.

Indirect evidence suggests that AE function is responsive to kinases [21,36,37]. Anion-exchange activity in the heart is stimulated by β -adrenergic agonists [38], purinergic agonists [39] and angiotensin II [40]. A role for tyrosine kinases is suggested by the observation that purinergic activation of anion-exchange activity in cardiomyocytes is inhibited by tyrosine kinase inhibitors [41]. Tyrosine-kinase-coupled receptors transmit some of their signal through SH3 proteins [42,43]. Interestingly, the cytoplasmic domain of full-length AE3 contains two potential SH3-binding sites, one of which is flanked by 11 consecutive glutamic acid residues, giving it the appearance of a charged-protein-binding site.

In the heart, AE3c is expressed at a much higher level than full-length AE3 [6]. It is possible that the unique domain of AE3c

serves as a scaffolding site for either peripheral or cytoskeletal proteins. In this case, the reduced activity of AE3c limits the total anion transport capacity of the cell, while increasing the number of plasma membrane binding sites. Precedent for AEs as plasma membrane attachment sites comes from the erythrocyte AE1 protein, whose cytoplasmic domain anchors the plasma membrane to the cytoskeleton through interaction with ankyrin [44].

The range of transport rates of AE isoforms (Table 1) has significance in those tissues, such as the heart, that express multiple AE isoforms. Northern blots show that cardiac tissue expresses AE1, AE2, full-length AE3 and AE3c [18]. Northern blots of rat heart mRNA indicate that the expression level of AE isoforms is: AE3c > AE3 $\gg$ AE2 > AE1 (results not shown). However, since each isoform was probed with a different cDNA, the quantification is not completely reliable. Since the anion-exchange activities of AE1 and AE2 are much greater than the AE3 isoforms, AE1 and AE2 may have a more significant role in the heart than is suggested by consideration of their mRNA expression levels only.

Results presented here demonstrate differential regulation of AEs by pH. In the present study we have confirmed that AE1 transport activity is relatively insensitive to changes in pH [19] and that AE2 transport activity is steeply negatively regulated by acid pH_i, consistent with its role in cellular acidification [20,21]. Previous researchers reported that when expressed in CHOP cells, AE2 was only 20% active at pH 7.3 and had approx. 8% of full activity at pH 6.9 [21]. In the present study we found that AE2 was 65% active at pH 7.3 and activity was reduced to 37% of maximum at pH 6.0. Half-maximal activity was attained at pH 7.4, which is close to the value of pH 7.5 found in CHOP cells. Therefore the pH of half-maximal activity is consistent between the two studies, but we found that AE2 was less sensitive to acid pH than the previous group. One possible explanation for the result is that in the present study both pH_i and pH_o were changed together, while in the CHOP cell study pH_o was held constant and pH_i was varied using pulses of NH₄Cl.

In contrast with our results, one previous study found that the anion-exchange activity of full-length AE3 is inhibited by acid pH_i [22]. In that study, Cl⁻/HCO₃⁻ exchange was quantified by the rate of change of pH_i, measured by following the rate of pH_i recovery in cells made acidic or alkaline. Thus the variable under study (the effect of pH_i) varied constantly during measurement of transport. By contrast, in the present study pH_i was clamped at a constant value during each transport assay. Therefore pH_i did not change during our measurement of transport activity (Figure 6), which allowed for better measurement of initial transport rates at different pH_i values. Alternatively, in the previous experiment only pH_i was varied, while in our experiments pH_i and pH_o were both simultaneously varied. We cannot rule out the possibility that AE3 transport is positively regulated by acid pH_o and negatively regulated by acid pH_i. The results of this study clearly show that transport by AE3 isoforms is essentially independent of changes in pH_o and pH_i. Although AE3c was previously shown in *X. laevis* oocytes to be a functional AE [7], the present study is the first examination of pH regulation of AE3c and AE3tr.

The retina is the only other tissue found to express both full-length AE3 and AE3c [5]. Interestingly, heart and retina both contain an extensive network of electrically coupled cells whose gap-junctional conductances are extremely sensitive to changes in pH_i. A 0.2–0.3 unit decrease in pH_i will lead to a 50% decrease in junctional conductance in the retina [45], and a decrease of pH_i of 0.3 will cause a 30–80% decrease in junctional conductance in the heart, with gap junctions composed of connexin 45 being the most affected [13]. It is clear that maintenance of

alkaline pH_i is of extreme importance in both retina and heart. We therefore propose that AE3 isoforms in the retina and heart are present to alkalize these cells by inward movement of bicarbonate. Our observation of AE3 activity under acid conditions underscores this possibility.

The central role of the CO₂/HCO₃⁻ buffer system for pH homeostasis is dependent on CA, an enzyme that catalyses the hydration–dehydration of CO₂/HCO₃⁻ [46]. The CA isoform present in cardiomyocytes is CAIV [47], which is linked *extracellularly* by a glycosylphosphatidyl inositol anchor to the plasma membrane [48]. CAIV has a significant role in facilitating the recovery of pH_i after reperfusion of ischaemic heart [47]. We attribute this to the movement of freely diffusible CO₂ out of the cell and its rapid conversion to HCO₃⁻ by CAIV. The extracellular HCO₃⁻ could then be exchanged for intracellular Cl⁻ by AE3 proteins, leading to cellular alkalization.

In the present study we have investigated the transport activity of both AE3 and AE3c and compared them to that of AE1 and AE2. Our results indicate that although a similar fraction of each AE is processed to the plasma membrane, the rate of anion transport by the AE3 isoforms is much lower than that of both AE1 and AE2. We have also noted that AE3c transports at a rate about half that of full-length AE3. This indicates that the longer N-terminal region of full-length AE3 confers some form of positive regulation on the protein. Our finding that anion transport by AE3 isoforms is essentially independent of changes in pH_o/pH_i indicates that these proteins may contribute to pH_i recovery following an acid load. Further studies are needed to determine which regulatory processes are important in the control of anion exchange by AE3 isoforms.

J.R.C. is a Scholar of the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. We thank Dr. Gary Schull for providing us with the cDNA clones for rat full-length AE3 and AE3c, and Dr. Ron Kopito for providing the AP3 antibody. Dr. James Young, Dr. Jocelyne Fujinaga and Deborah Merriam provided helpful comments on the manuscript. This work was supported by a grant-in-aid from the Heart and Stroke Foundation of Alberta.

REFERENCES

- 1 Cabantchik, Z. I. and Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226
- 2 Kopito, R. R., Lee, B. S., Simmons, D. M., Lindsey, A. E., Morgans, C. W. and Schneider, K. (1989) *Cell* **59**, 927–937
- 3 Alper, S. L., Kopito, R. R., Libresco, S. M. and Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 17092–17099
- 4 Linn, S. C., Kudrycki, K. E. and Schull, G. E. (1992) *J. Biol. Chem.* **267**, 7927–7935
- 5 Kobayashi, S., Morgans, C. W., Casey, J. R. and Kopito, R. R. (1994) *J. Neurosci.* **14**, 6266–6279
- 6 Linn, S. C., Askew, G. R., Menon, A. G. and Shull, G. E. (1995) *Circ. Res.* **76**, 584–591
- 7 Yannoukakos, D., Stuart-Tilley, A., Fernandez, H. A., Fey, P., Duyk, G. and Alper, S. L. (1994) *Circ. Res.* **75**, 603–614
- 8 Ek-Vitorin, J. F., Calero, G., Morley, G. E., Coombs, W., Taffet, S. M. and Delmar, M. (1996) *Biophys. J.* **71**, 1273–1284
- 9 Busa, W. B. and Nuccitelli, R. (1984) *Am. J. Physiol.* **246**, R409–R438
- 10 Isfort, R. J., Cody, D. B., Asquith, T. N., Ridder, G. M., Stuard, S. B. and LeBoeuf, R. A. (1993) *Eur. J. Biochem.* **213**, 349–357
- 11 Reynolds, J. E., Li, J. and Eastman, A. (1996) *Cytometry* **25**, 349–357
- 12 Orchard, C. H. and Kentish, J. C. (1990) *Am. J. Physiol.* **27**, C967–C981
- 13 Hermans, M. M., Kortekaas, P., Jongsma, H. J. and Rook, M. B. (1995) *Pfluegers Arch.* **431**, 138–140
- 14 Sardet, C., Franchi, A. and Pouyssegur, J. (1989) *Cell* **56**, 271–280
- 15 Romero, M. F., Hediger, M. A., Boulpaep, E. L. and Boron, W. F. (1997) *Nature (London)* **387**, 409–413
- 16 Garcia, C. K., Brown, M. S., Pathak, R. K. and Goldstein, J. L. (1995) *J. Biol. Chem.* **270**, 1843–1849
- 17 Pucéat, M., Korichneva, I., Cassoly, R. and Vassort, G. (1995) *J. Biol. Chem.* **270**, 1315–1322

- 18 Kudrycki, K. E., Newman, P. R. and Schull, G. E. (1990) *J. Biol. Chem.* **265**, 462–471
- 19 Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* **103**, 61–223
- 20 Humphreys, B. D., Jiang, L., Chernova, M. N. and Alper, S. L. (1994) *Am. J. Physiol.* **267**, C1295–C1307
- 21 Jiang, L., Stuart-Tilley, A., Parkash, J. and Alper, S. L. (1994) *Am. J. Physiol.* **267**, C845–C856
- 22 Lee, B. S., Gunn, R. B. and Kopito, R. R. (1991) *J. Biol. Chem.* **266**, 11448–11454
- 23 Sarkar, G. and Sommer, S. S. (1990) *BioTechniques* **8**, 404–407
- 24 Fierke, C. A., Krebs, J. F. and Venters, R. A. (1991) in *International Workshop on Carbonic Anhydrase: From Biochemistry and Genetics to Physiology and Clinical Medicine* (Botre, F., Gros, G. and Storey, B. T., eds.), pp. 22–36, V. C. H., Weinheim, Germany
- 25 Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977) *J. Gen. Virol.* **52**, 59–72
- 26 Ruetz, S., Lindsey, A. E., Ward, C. L. and Kopito, R. R. (1993) *J. Cell Biol.* **121**, 37–48
- 27 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 28 Tang, X. B., Fujinaga, J., Kopito, R. and Casey, J. R. (1998) *J. Biol. Chem.* **273**, 22545–22553
- 29 Casey, J. R., Ding, Y. and Kopito, R. R. (1995) *J. Biol. Chem.* **270**, 8521–8527
- 30 Thomas, J. A., Buchsbaum, R. N., Zimniak, A. and Racker, E. (1979) *Biochemistry* **18**, 2210–2218
- 31 Silva, N. L., Wang, H., Harris, C. V., Singh, D. and Fliegel, L. (1997) *Pfluegers Arch.* **433**, 792–802
- 32 Roos, A. and Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–434
- 33 Lindsey, A. E., Schneider, K., Simmons, D. M., Baron, R., Lee, B. S. and Kopito, R. R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5278–5282
- 34 Illsley, N. P. and Verkman, A. S. (1987) *Biochemistry* **26**, 1215–1219
- 35 Zhang, Y., Chernova, M. N., Stuart-Tilley, A. K., Jiang, L. and Alper, S. L. (1996) *J. Biol. Chem.* **271**, 5741–5749
- 36 Olsnes, S., Tonessen, T. I. and Sandvig, K. (1986) *J. Cell Biol.* **102**, 967–971
- 37 Baggio, B., Bordin, L., Clari, G., Gambaro, G. and Moret, V. (1993) *Biochim. Biophys. Acta* **1148**, 157–160
- 38 Désilets, M., Pucéat, M. and Vassort, G. (1994) *Circ. Res.* **75**, 862–869
- 39 Pucéat, M., Clément, O. and Vassort, G. (1991) *J. Physiol. (London)* **444**, 241–256
- 40 Grace, A. A. and Vandenberg, J. I. (1993) *Circulation* **88**, I-331
- 41 Puceat, M., Roche, S. and Vassort, G. (1998) *J. Cell Biol.* **141**, 1637–1646
- 42 Pawson, T. and Gish, G. D. (1992) *Cell* **71**, 359–362
- 43 Pawson, T. (1994) *Adv. Cancer Res.* **64**, 87–110
- 44 Low, P. S. (1986) *Biochim. Biophys. Acta* **864**, 145–167
- 45 DeVries, S. H. and Schwartz, E. A. (1989) *J. Physiol. (London)* **414**, 351–375
- 46 Maren, T. H. (1967) *Physiol. Rev.* **47**, 595–781
- 47 Vandenberg, J. I., Carter, N. D., Bethell, H. W., Nogradi, A., Ridderstrale, Y., Metcalfe, J. C. and Grace, A. A. (1996) *Am. J. Physiol.* **271**, C1838–C1846
- 48 Waheed, A., Zhu, X. L. and Sly, W. S. (1992) *J. Biol. Chem.* **267**, 3308–3311

Received 3 June 1999/11 August 1999; accepted 14 September 1999