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RESEARCH PAPER

Antibodies against the cardiac sodium/bicarbonate co-transporter (NBCe1) as pharmacological tools

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BACKGROUND AND PURPOSE
Na^+/HCO_3^- co-transport (NBC) regulates intracellular pH (pH_i) in the heart. We have studied the electrogenic NBC isoform NBCe1 by examining the effect of functional antibodies to this protein.

EXPERIMENTAL APPROACH
We generated two antibodies against putative extracellular loop domains 3 (a-L3) and 4 (a-L4) of NBCe1 which recognized NBCe1 on immunoblots and immunostaining experiments. pH_i was monitored using epi-fluorescence measurements in cat ventricular myocytes. Transport activity of total NBC and of NBCe1 in isolation were evaluated after an ammonium ion-induced acidosis (expressed as H^+ flux, J_H, in $mmol \cdot L^{-1}$ min^{-1} at pH_i 6.8) and during membrane depolarization with high extracellular potassium (potassium pulse, expressed as ΔpH_i) respectively.

KEY RESULTS
The potassium pulse produced a pH_i increase of 0.18 ± 0.006 (n = 5), which was reduced by the a-L3 antibody (0.016 ± 0.019). The a-L-3 also decreased J_H by 50%. Surprisingly, during the potassium pulse, a-L4 induced a higher pH_i increase than control,(0.25 ± 0.018) whereas the recovery of pH_i from acidosis was faster (J_H was almost double the control value). In perforated-patch experiments, a-L3 prolonged and a-L4 shortened action potential duration, consistent with blockade and stimulation of NBCe1-carried anionic current respectively.

CONCLUSIONS AND IMPLICATIONS
Both antibodies recognized NBCe1, but they had opposing effects on the function of this transporter, as the a-L3 was inhibitory and the a-L4 was excitatory. These antibodies could be valuable in studies on the pathophysiology of NBCe1 in cardiac tissue, opening a path for their potential clinical use.

Abbreviations
βi, intrinsic buffering capacity; AE1, Cl^-/HCO_3^- exchanger isoform 1; AE3, Cl^-/HCO_3^- exchanger isoform 3; a-L3, antibody against putative extracellular loop domain 3 of NBCe1; a-L4, antibody against putative extracellular loop domain 4 of NBCe1; AP, cardiac action potential; APD, action potential duration; BCECF, 2′,7′-Bis-(2-carboxyethyl)-5,6-carboxyfluorescein; CAII, carbonic anhydrase II; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; EC3, extracellular loop 3; EC4, extracellular loop 4; eNBC, electrogenic Na^+/HCO_3^- co-transporter; GST, glutathione S-transferase; NBC, Na^+/HCO_3^- co-transporter; NBCe1, electrogenic Na^+/HCO_3^- co-transporter isoform 1; NBCe2, electrogenic Na^+/HCO_3^- co-transporter isoform 2; NBCn1, electroneutral Na^+/HCO_3^- co-transporter isoform 1; NHE, Na^+-H^+ anti-porter; N-I serum, non-immune serum; nNBC, electroneutral Na^+/HCO_3^- co-transporter; pH_i, intracellular pH; SITS, 4-acetamido-4-isothiocyanatostilbene-2,2′-disulphonic acid

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Introduction

Intracellular pH (pH_i) modulates excitation and contraction in the heart (Bountra and Vaughan-Jones, 1989; Bers, 2001). Ion transporters facilitate the fine control of cardiac pH_i and attenuate the adverse effects of large changes in pH_i (Vaughan-Jones *et al.*, 2009). The major transporters responsible for acid extrusion are the Na^+-H^+ anti-porter (NHE) and Na^+-HCO_3^- co-transporters (NBC), which transport H^+ out of and HCO_3^- into the cell respectively (Dart and Vaughan-Jones, 1992; Karmazyn and Moffat, 1993). In the absence of HCO_3^-, the only acid extruding mechanism is the widely studied NHE. In medium with bicarbonate, like the blood, both mechanisms, NHE and NBC, are equally operative at a pH_i close to basal (Lagadic-Gossmann *et al.*, 1992; Camilion de Hurtado *et al.*, 1995; Le Prigent *et al.*, 1997; Vaughan-Jones *et al.*, 2006; 2009).

Distinct NBC isoforms coexist in the mammalian myocardium, the electrogenic isoforms, NBCe1 (or NBC1) (Romero *et al.*, 1997; Choi *et al.*, 1999) and NBCe2 (or NBC4) (Pushkin *et al.*, 2000; Virkki *et al.*, 2002), with a stoichiometry of 1 Na^+:2 HCO_3^-, and the electroneutral isoform NBCn1 (or NBC3), with a stoichiometry of 1 Na^+:1 HCO_3^-. The electrogenic NBCe1 and NBCe2 are encoded by the *SLC4A4* gene (Choi *et al.*, 1999), and the *SLC4A5* gene (Pushkin *et al.*, 2000; Sassani *et al.*, 2002), respectively, while NBCn1 is encoded by the *SLC4A7* gene (Choi *et al.*, 2000). In our laboratory, we have determined the influence of the electrogenic NBC (eNBC) in the configuration of the rat and cat ventricular action potential (AP), generating an anionic repolarizing current, which produces an AP shortening of approximately 25% (Aiello *et al.*, 1998; Villa-Abrille *et al.*, 2007). The electrogenic isoform involved in this effect was not, however, determined in our previous studies.

In the present study, we focus our attention on NBCe1, by examining the effect of isoform-specific antibodies. These polyclonal antibodies were produced in rabbits injected with fusion proteins, containing the amino-acid sequence of the extracellular loop 3 (EC3) and extracellular loop 4 (EC4) of the putative topological structure of NBCe1 (Alvarez *et al.*, 2003). As these antibody epitopes are localized on the extracellular side of NBCe1, we hypothesized that antibodies selectively directed against them could be able to interfere with NBCe1 transport function and therefore, might be employed as pharmacological tools. Herein, we present evidence for inhibition and stimulation of NBCe1 transport activity with antibodies selectively directed against EC3 (a-L3) and EC4 (a-L4), respectively, validating our hypothesis.

Methods

Glutathione-S-transferase (GST) fusion protein construction

Bacterial expression constructs encoding GST-fusion protein consisting of the cDNA for GST fused to cDNA corresponding to the third (EC3) or fourth (EC4); putative extracellular loops of human NBCe1 were constructed as previously described (Alvarez *et al.*, 2003).

Protein expression

Expression constructs for NBCe1b, the human cardiac splicing variant of NBCe1 (Alvarez *et al.*, 2003) and human NBCn1 (Loiselle *et al.*, 2003), have been described previously. Expression construct for rat NBCe2 (NBC4c) was a gift from Dr Jeppe Praetorius (Aarhus University, Denmark). Human HEK293 cells were individually transfected with NBCe1b, NBCe2 or NBCn1, as indicated, using the calcium phosphate method (Ruetz *et al.*, 1993). Cells were grown at 37°C in an air/CO_2 (19:1) environment in Dulbecco's modified Eagle's medium supplemented with 10% (v/v).

Antibody preparation

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Male New Zealand rabbits (San Cayetano, Monte Grande, Buenos Aires, Argentina) (3 months old) were immunized subcutaneously with 1 mg of fusion protein in Freund's complete adjuvant followed by a twice immunization at monthly intervals with 1 mg of fusion protein in Freund's incomplete adjuvant. The animals were exsanguinated, and crude sera with the designated antibodies anti-NBCe1 loop 3 (a-L3) and anti-NBC1 loop 4 (a-L4), respectively, were obtained. Freund's complete adjuvant without fusion protein was injected to rabbits to generate non-immune serum (N-I serum).

Immunodetection

Two days post-transfection, cells were washed in PBS buffer (in $mmol \cdot L^{-1}$: 140 NaCl, 3 KCl, 6.5 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.5) and cell lysates were prepared by the addition of 150 µL SDS-PAGE sample buffer to 60 mm Petri dish. Samples of untransfected or transfected HEK293, or samples of cat ventricular lysates (50 µg protein), were resolved by SDS-PAGE on 7.5 acrylamide gels. Proteins were transferred to PVDF membranes, and then incubated with rabbit anti-NBC1 antibody (Millipore, Temecula, CA, USA; 1:1000 dilutions), rabbit anti-NBCe1 loop 3 (a-L3) antibody (1:500 dilutions), rabbit anti-NBCe1 loop 3 (a-L4) antibody (1:500 dilutions), rabbit anti-NBCe2 antibody (ab99131 Abcam, Cambridge, MA, USA; 1:2000 dilutions), rabbit anti-NBCn1 antibody (1:2000 dilutions) (Loiselle *et al.*, 2003) or N-I serum (1:500 dilutions). Membranes were blocked with 5% non-fat milk. Immunoblots were incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase and visualized using the ECL reagent and a Chemidoc Image Station (Bio-Rad, Hercules, CA, USA).

To demonstrate the specificity of the immunoreactivity, duplicate samples were submitted to the same protocol except that the primary antibody was incubated for 1 h in the presence of the EC3 and EC4 fusion proteins (1 µg ml^{-1} final concentration) used to generate the antiserum.

Preparation of ventricular myocytes

Cat ventricular myocytes (San Cayetano, Monte Grande, Buenos Aires, Argentina) were isolated according to the techniques previously described (Aiello and Cingolani, 2001).

pH_i measurements

pH_i was measured in single myocytes with an epi-fluorescence system (Ion Optix, Milton, MA, USA), using the 2′,7′-Bis-(2-

carboxyethyl)-5,6-carboxyfluorescein (BCECF) technique, as previously described (De Giusti *et al.*, 2008). Briefly, myocytes were incubated at room temperature for 30 min with 10 μmol·L^{-1} BCECF-acetoxymethylester followed by 30 min washout. Dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon TE 2000-U, Nikon, Tokyo, Japan) and continuously superfused with a solution, containing (mmol·L^{-1}) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 Cl_2Mg, 1.35 Cl_2Ca, 10 glucose, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. The myocytes were stimulated via two-platinum electrodes on either side of the bath at 0.5 Hz. Dual excitation (440 and 495 nm) was provided by a 75 W xenon arc lamp and transmitted to the myocytes. Emitted fluorescence was collected with a photomultiplier tube equipped with a band-pass filter centred at 535 nm. The 495 to 440 nm fluorescence ratio was digitized at 10 kHz (ION WIZARD fluorescence analysis software, IonOtix, Milton, MA, USA). At the end of each experiment, the fluorescence ratio was converted to pH by calibrations using the high K^+-nigericin method (Perez *et al.*, 1995).

Ammonium pulse

As described previously, the experiments were performed in HCO_3^- buffered solution. Under these conditions, both pH_i alkalinizing systems are operative, NHE and NBC. In order to examine only the NBC activity, all the experiments were performed in the presence of the NHE inhibitor HOE642 (cariporide; 10 μmol·L^{-1}). Total NBC activity was assessed by evaluating the pH_i recovery from an ammonium pre-pulse-induced acute acid load. Transient (3 min) exposure of myocytes to 20 mmol·L^{-1} NH_4Cl was used for this purpose. The dpH_i/dt at each pH_i, obtained from an exponential fit of the recovery phase, was analysed to calculate the net H^+ efflux (J_H), then $J_H = \beta_{tot}\ dpH_i/dt$, where β_{tot} is total intracellular buffering capacity. β_{tot} was calculated by the sum of the intracellular buffering due to CO_2 (β_{CO2}) plus the intrinsic buffering capacity (βi). β_{CO2} was calculated as $\beta_{CO2} = 2.3\ [HCO_3^-]_i$, where $[HCO_3^-]_i = [HCO_3^-]_o\ 10^{pHi-pHo}$ (Leem *et al.*, 1999; Roos and Boron, 1981). βi of the myocytes was measured by exposing cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. pH_i was allowed to stabilize in Na^+-free solution before application of NH_4Cl. βi was calculated from the equation $\beta i = \Delta[NH_4^+]i/\Delta pH_i$ and referred to the mid-point values of the measured changes in pH_i. βi at different levels of pH_i was estimated from the least squares regression lines βi versus pH_i plots (Supporting Information Figure S1).

Potassium pulse

To investigate the eNBC activity in isolation, we performed a potassium pulse. Increasing isotonically extracellular K^+ $[K^+]_o$ from 5 to 45 mmol·L^{-1} produced a depolarization of approximately 60 mV that enhanced the eNBC activity and in turn elevated pH_i. The high K^+ was applied for 14 min and during this period the pH_i was recorded. The HCO_3^--buffered solution used in the K^+-induced depolarization experiments contained (mmol·L^{-1}): 118 NaCl, 5 KCl, 1 $MgSO_4$, 0.35 NaH_2PO_4, 10 glucose, 40 choline chloride, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. K^+-induced depolarization was assessed by replacing 40 mmol·L^{-1} choline chloride with 40 mmol·L^{-1} KCl, maintaining ionic strength.

Immunostaining of cat cardiac myocytes and analysis by confocal microscopy

Freshly single dissociated adult cat myocytes were plated onto 22 × 22 mm laminin (30 μg mL^{-1})-coated glass coverslips and incubated at 37°C for 45 min to allow attachment. Cells were rinsed with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature, followed by methanol fixation-permeabilization [ice-cold 100% (v/v) methanol, 5 min at –20°C]. Myocytes were then washed with PBS and permeabilized with 0.1% Triton X-100 (v/v) in PBS for 15 min at room temperature. After washing (2 × 5 min with PBS) and blocking (5% BSA in PBS, 20 min) were completed, the cells were incubated with primary antibodies (1 h at room temperature, in a humidified chamber), washed (3 × 5 min in PBS containing 0.2% gelatin) and incubated with secondary antibody. Primary rabbit polyclonal a-L3, rabbit polyclonal a-L4 antibody and N-I serum were used at 1:100 dilution. Secondary chicken anti-rabbit conjugated to Alexa fluor 488 was used at 1:200 dilutions. Coverslips were washed three times in PBS containing 0.2% gelatin and twice in PBS, and mounted and viewed with a confocal microscope. Immunostained cells were mounted in Prolong anti-fade solution (Molecular Probes, Eugene, OR, USA) and imaged with an Olympus Bx61 laser scanning confocal microscope imaging system. Images were collected with an oil immersion ×60 1.4 objective (numerical aperture 0.2, plan Apochromat, Zoom 1.5×). Images were captured in a sequential manner, and analyzed with the Fluoview 3.3 Software (Olympus, Madison, WI, USA).

Double immunostaining of cat cardiac myocytes

Isolated adult cat cardiomyocytes were fixed to laminin-coated coverslips and permeabilized as described above. Myocytes were incubated with a combination of a-L3 antibody and goat polyclonal anti-vinculin antibody (N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a combination of rabbit a-L4 antibody and goat anti-vinculin antibody. Combined primary antibodies were used at 1:100 dilutions. Secondary chicken anti-rabbit conjugated to Alexa Fluor 488 and chicken anti-goat conjugated to Alexa fluor 594 was used at 1:200 dilutions. Images were captured and analysed as explained previously.

Patch-clamp recordings

The nystatin-perforated whole-cell configuration of the patch-clamp technique was used for current-clamp recordings with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA). Patch pipettes were pulled with a PP-83 puller (Narishige, Tokyo, Japan) and fire polished with an MF-83 Microforge (Narishige) to a final resistance of 0.5–1 MΩ when filled with a control pipette solution. Membrane voltage (sampling rate = 1 kHz; low pass filter = 1 kHz) was digitally recorded directly to hard disc via an analog-to-digital convertor (Digidata 1200, Axon Instruments) interfaced with a computer running pClamp software (Axon Instruments). A pacing rate of 0.2 Hz was applied. Data analysis was performed with pClamp (Clampfit, Axon Instruments). An Ag/AgCl wire directly in contact with the extracellular solution was used as reference electrode. As the pipette potential was nulled in external solution, all current-

clamp tracings required corrections for junction potential. This was accomplished by filling 20 pipettes with standard internal solution. They were then nulled in internal solution, and the difference in potential on immersion in external solution was recorded. The measured junction potential value was consistently –10 mV, and this value was used to correct all current-clamp data and voltage-clamp protocols. There were no significant differences in the value of junction potential among all the external solutions used in the present work. Bath solution was the same as that used for the pH_i measurements in the ammonium pulse experiments. The nystatin pipette solution contained (in mmol·L^{-1}): K gluconate 130, KCl 10, NaCl 5, $MgCl_2$ 0.5, EGTA 1, HEPES 10 and nystatin 0.3 mg ml^{-1}. The pH was titrated to 7.2 with KOH.

Statistics

Data were expressed as means ± SEM and were compared with Students's *t*-test or One-way ANOVA followed by Student–Newman–Keuls *post hoc* test. A value of $P < 0.05$ was considered statistically significant (two-tailed test).

Materials

BCECF (Invitrogen, Buenos Aires, Argentina) HOE642 S0859 (Sanofi-Aventis, Frankfurt, Germany).

Results

Expression and localization of NBC1 in cat myocardium

Expression of Na^+/HCO_3^- co-transporters NBCe1, NBCn1 and NBCe2 has been previously observed in heart tissue (Choi *et al.*, 1999; Pushkin *et al.*, 1999; 2000). Expression of NBCe1 protein has been documented in mammalian heart (Khandoudi *et al.*, 2001). Expression of NBCn1 and NBCe2, however, has only been detected at the mRNA level in the heart, using Northern blot and reverse transcription RT-PCR analysis (Ishibashi *et al.*, 1998; Pushkin *et al.*, 1999; Choi *et al.*, 2000). We examined the expression of NBCe1 protein in the cat myocardium, using different antibodies. Antibodies were raised against fusion proteins corresponding to putative third (EC3) and fourth (EC4) extracellular loops of human NBCe1 and used to detect NBCe1 in isolated ventricles of cat heart. Amino acid sequence alignment of human sequences of SLC4 family members, AE and NBC families, are presented in Figure 1A. The alignments of putative EC3 and EC4 regions of bicarbonate transport proteins, although showing clusters of conserved sequence, present enough non-conserved amino acids to constitute dissimilar antigen determinant regions, minimizing the risk of inducing the generation of antibodies that could produce cross-reaction between the different transporters. Immunoblots of cat ventricles, probed using a specific commercial anti-NBCe1 antibody (Millipore; Figure 1B), a-L4 (Figure 1C) and a-L3 (Figure 1D) demonstrated a band with a molecular mass of ~130 kDa, corresponding to NBCe1 (Alvarez *et al.*, 2003). A band with a similar molecular mass was detected in lysates of HEK293 cells transfected with NBCe1 used as controls (Figure 1B–D). Cat cardiac NBCe1 appears to be slightly larger than NBCe1 transfected in HEK293 cells, possibly due to glycosylation of the protein in myocardium. No immunoreactivity was found in non-transfected HEK293 cells (Figure 1B–D), or on parallel blots incubated with non-immune serum (N-I serum) (Figure 1E). Other non-specific bands were detected with the commercial anti-NBCe1, as well as with the serum containing a-L3 or a-L4. We did not perform additional assays to investigate this matter. The existence of different oligomeric (dimeric, tetrameric) states of NBCe1 in transfected HEK293 cells has been demonstrated before (Kao *et al.*, 2008). A single band of approximately 250 kDa is observed in lysates of NBCe1-transfected cells (Figure 1D), which may therefore represent a dimer.

As EC3 and EC4 loops of NBCe1 share some amino acid identity with homologous regions in other NBC proteins (Figure 1A) and NBCe2 and NBCn1 gene products are also proposed to be expressed in mammalian cardiac myocytes, it is essential to establish the selectivity of a-L3 and a-L4 for NBCe1. Figure 2A shows that a-L3 or a-L4 failed to detect NBCn1 (135 kDa) or NBCe2 (126 kDa) in lysates of HEK cells transfected with these NBC isoforms on immunoblots. Using specific antibodies against these NBC isoforms, we confirmed in parallel blots that the HEK cells used in Figure 2A were efficiently transfected with NBCn1 or NBCe2 (Figure 2A, inset). Note that an immunoreactive band is present in lysates of non-transfected HEK cells, suggesting that NBCn1 is endogenously expressed in these cells (Figure 2A, inset).

To determine whether the a-L3 or a-L4 sera specifically target the EC3 or EC4 loops of NBCe1, we performed competition experiments. These sera were co-incubated with the GST fusion proteins used to generate them. As shown in Figure 2B, the fusion proteins pre-adsorbed immunoreactivity from a-L3 and a-L4, because no band corresponding to NBCe1 was detected in lysates of NBCe1-transfected HEK cells on immunoblots probed with the fusion-protein pre-adsorbed sera.

Other members of the SLC4 family, AE1 and AE3, have been localized to the sarcolemma (SL), and to the SL and T-tubules system of cardiomyocytes respectively (Alvarez *et al.*, 2007). In order to study the localization of NBC1, we performed confocal microscopy studies. Antibodies against NBCe1 revealed labelling surrounding the cat isolated cardiomyocytes, consistent with sarcolemmal staining (Figure 3A). In addition, NBCe1 staining shows intracellular localization, with invaginations that run towards the centre of the cardiomyocytes. Such a distribution is consistent with the presence of NBCe1 in the sarcolemma and along the transverse tubular system (T-tubules), as suggested by the longitudinal bands. No immunolabelling was seen with the N-I serum, in isolated cat cardiomyocytes (Figure 3A).

The identity of the intracellular structures containing NBCe1 was further explored in double-labelling experiments of freshly isolated cat cardiomyocytes (Figure 3B). NBCe1 protein was found with transverse rib-like pattern, suggesting specific location of this protein in the T-tubules, an invagination of the SL at the level of the Z band, extending deep into the cardiomyocytes. NBCe1 showed intense co-localization with vinculin, a cell-matrix focal adhesion molecule present in the T system, as observed in the merged image (yellow, Figure 3B).

We conclude that the electrogenic NBCe1 protein was expressed in cat heart muscle, as detected by immunoblots. NBCe1 has a restricted pattern of localization in isolated cat

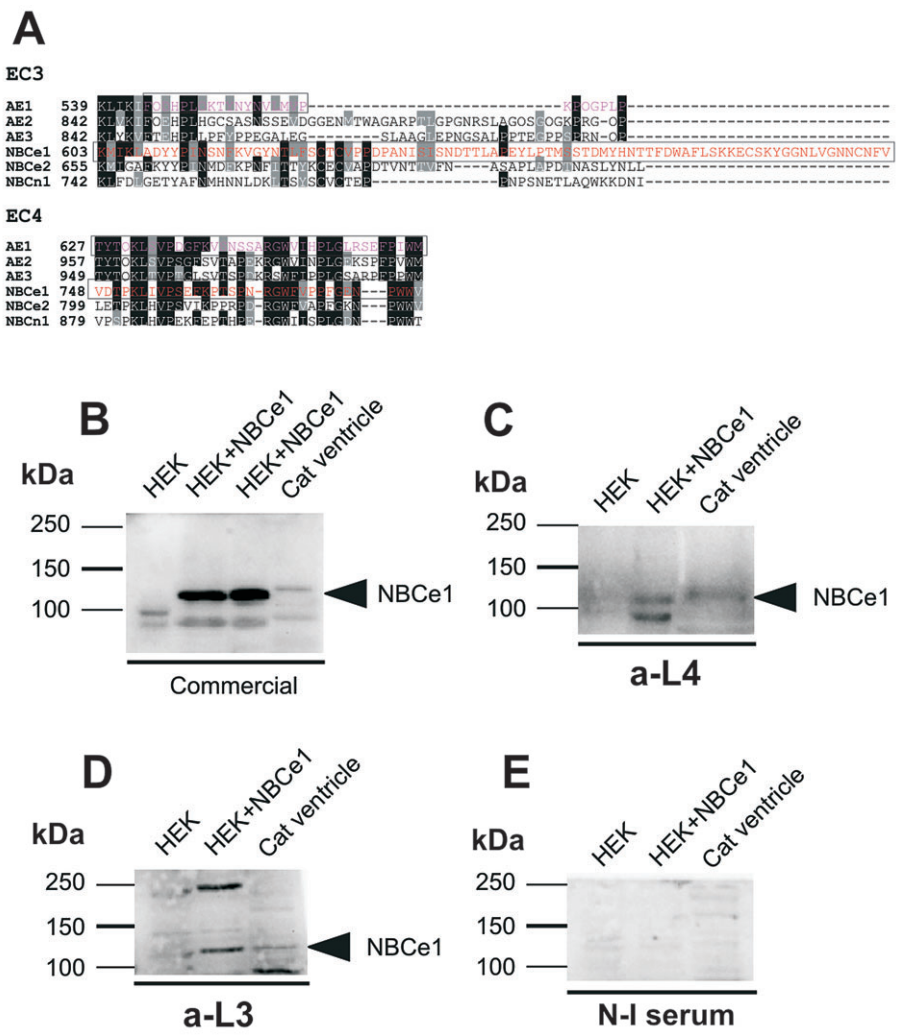

Figure 1

Alignment of amino acid sequences of bicarbonate transport proteins and expression of NBCe1 protein in cat ventricles. (A) Alignment of amino acid sequences of bicarbonate transporter EC3 and EC4. Sequence conservation is indicated by black rectangles (identical amino acids) and grey rectangles (similar amino acids). Pink colour indicates EC3 and EC4 loops predicted from AE1 (Tang *et al.*, 1999; Zhu *et al.*, 2003). Orange colour indicates amino acids used for a-L3 and a-L4 construction. (B–E) Lysates of HEK293 cells; HEK293 cells transfected with NBCe1 or cat ventricles (50 μg) were resolved by SDS-PAGE on 8% polyacrylamide gels, transferred to PVDF membrane and probed for NBC1 with a commercial antibody against NBC1 N-terminus (amino acids 338–391) (B), a-L3 (C), a-L4 (D) or N-I serum (E). Position of NBCe1 is indicated with arrowheads.

cardiomyocytes, being specifically localized to SL and T-tubules. On the basis of its function and localization, a fundamental role for NBCe1 in cardiomyocytes excitation–contraction coupling is possible.

Determination of eNBC activity with the potassium pulse

Myocytes were exposed to a high extracellular K^+ solution (45 mmol·L^{-1}) to induce hyperkalemic depolarization of membrane potential, which resulted in an alkalization that was reversed upon washout of the high K^+ solution (Figure 4A). Analysis of these experiments revealed that this increase in pH_i was blocked by pretreatment of the cells with the recently reported NBC blocker S0859 (Ch'en *et al.*, 2008), but not by the NHE inhibitor HOE642, indicating that this effect is mediated by selective activation of eNBC (Figure 4B).

To evaluate further the specificity of the depolarization assay to determine eNBC activity, experiments in the absence of sodium or bicarbonate were performed. The pH_i increases induced by the high extracellular K^+ solution were abolished by removing Na^+ and HCO_3^- (Supporting Information Figure S2), indicating that the pH_i changes arose from the electrochemical activation of eNBC.

The enzyme carbonic anhydrase II (CAII) carries rapid CO_2/HCO_3^- interconversion and facilitates membrane NBCe1-mediated HCO_3^- transport (Alvarez *et al.*, 2003). Inhibition of CAII is commonly achieved with sulphonamides or their derivatives. However, recently, some non-sulphonamides, such as HOE642, have been reported to inhibit the enzyme (Villafuerte *et al.*, 2007). In our experiments, HOE642 did not affect maximal NBCe1-mediated alkalization after high potassium depolarization, although it

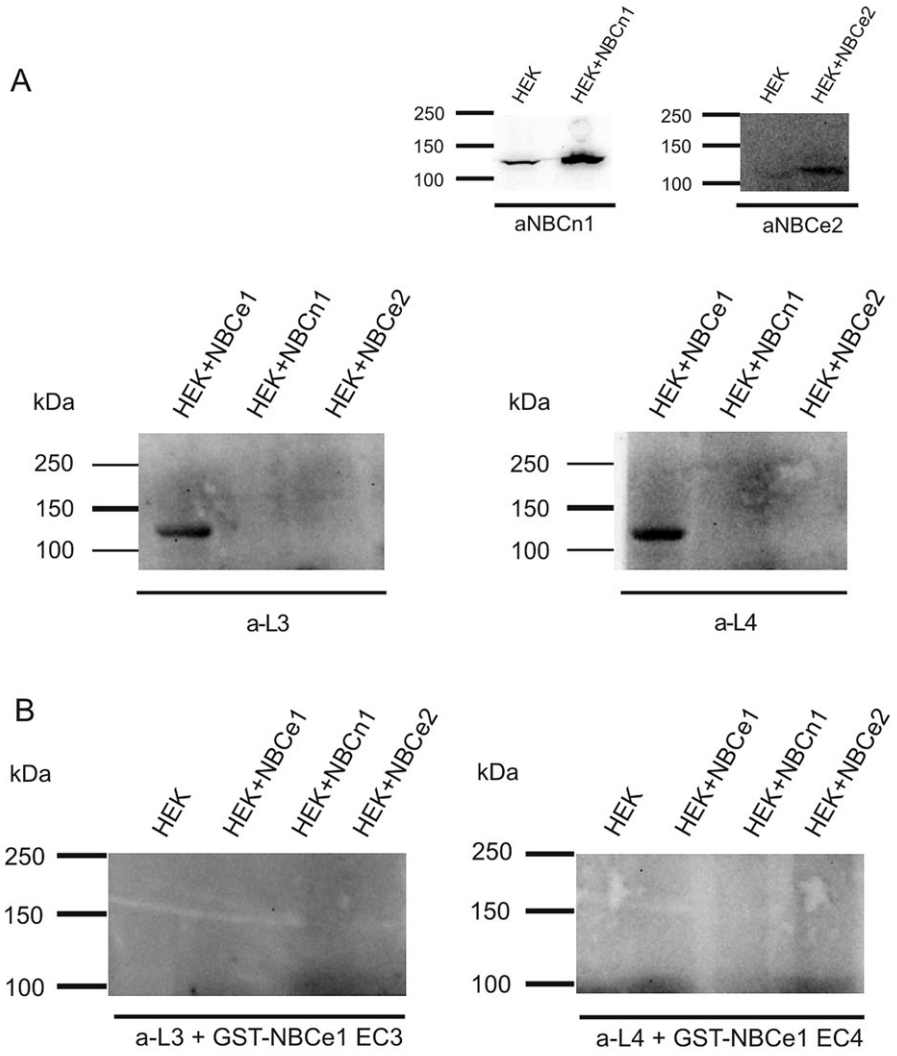

Figure 2

Detection of human Na^+/HCO_3^- co-transporter NBCe1 protein in membrane preparations from HEK293 cells. Cell lysates were prepared from untransfected HEK293 cells or HEK293 cells individually transfected with NBCe1, NBCn1 or NBCe2 cDNA. Proteins were resolved by SDS-PAGE and then detected by immunoblotting. Blots were pre-incubated with a-L3 or a-L4 antiserum (final dilution 1:500) either in absence (A) or presence (B) of soluble NBCe1 fusion proteins (final concentration 1 μg ml^{-1}) used to generate antisera, followed by incubation with donkey anti-rabbit IgG conjugated with horseradish peroxidase (HRP). Inset blots (above) were pre-incubated with a-NBCn1 antibodies (left, 1:2000), or pre-incubated with a-NBCe2 antibodies (right, 1:2000) followed by HRP-conjugated donkey anti-rabbit antibodies. Labelled proteins were detected by enhanced chemiluminescence. Positions of pre-stained molecular mass markers are indicated at left.

did slow the time-course of alkalization achieved by NBCe1. For CAII, the IC_{50} values for HOE642 are 12.4 μmol·L^{-1} and 60 μmol·L^{-1}, for pure CAII or cardiac CA homogenate, respectively (Villafuerte *et al.*, 2007), which are in the range of the dose we have used here (10 μmol·L^{-1}). Thus some inhibition of NBCe1-mediated HCO_3^- -transport by this drug may therefore be secondary to inhibition of CAII.

Opposing functional effects of a-L3 and a-L4 on NBCe1 activity evaluated with the potassium pulse

Effects of the antibodies against extracellular loops of NBCe1 were evaluated with the potassium pulse approach in isolated cat cardiomyocytes. The potassium pulse produced a control (N-I serum dilution 1/500) pH_i increase of 0.18 ± 0.01 pH units (n = 5) that was abolished by the a-L3 (serum dilution 1/500), indicating that NBCe1 is the predominant functional electrogenic NBC present in cat ventricular myocytes (Figure 5). Surprisingly, during the potassium pulse, the a-L4 (serum dilution 1/500) induced a higher pH_i increase than control (Figure 5). This stimulatory effect of a-L4 was not produced in the absence of sodium or bicarbonate or in the presence of S0859 (Supporting Information Figure S3), revealing a specific action on NBCe1.

In order to confirm that a-L3 or a-L4 sera did not exert non-specific effects, we performed functional competition

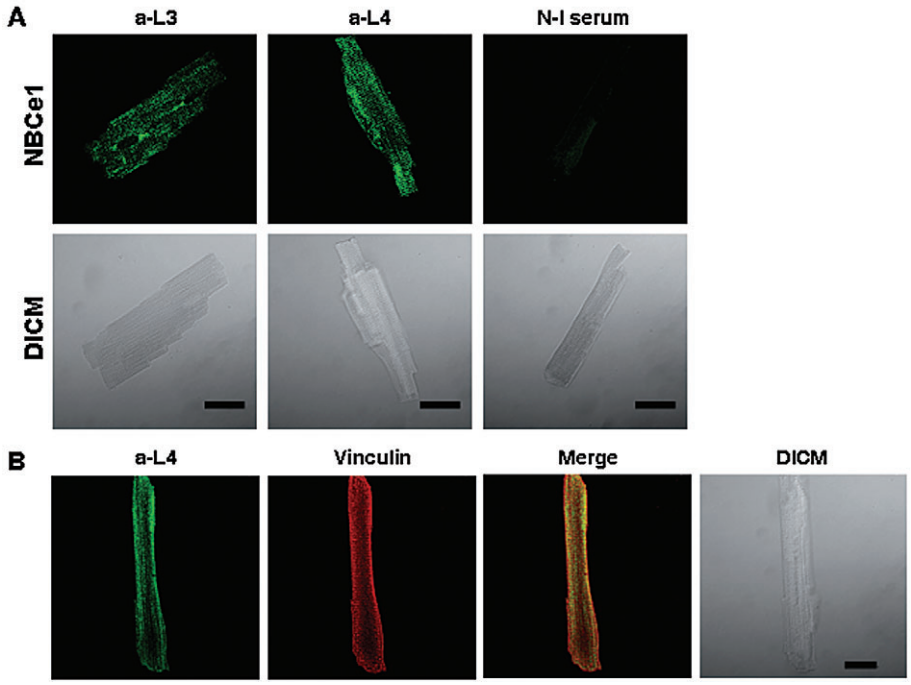

Figure 3

Localization of NBCe1 in cat ventricular myocytes. Confocal immunofluorescence analysis of the distribution of NBCe1 in cat myocytes. (A) Freshly isolated cat cardiomyocytes were stained with rabbit a-L3 antibody, or rabbit a-L4, or non-immune serum, as indicated in the panels. Images were collected with an Olympus Bx61 laser-scanning confocal microscope, with an oil immersion ×60/1.4 objective. Scale bars are 30 μm. (B) cat cardiomyocytes were double-stained with rabbit a-L4 (green) and goat anti-vinculin antibody (red), as indicated in the panels. Immunofluorescence signals were visualized by an Alexa fluor 488-conjugated anti-rabbit IgG antibody (green, 1:200 dilution), and Alexa fluor 594-conjugated anti-goat IgG antibody (red, 1:200 dilution). Co-localization of NBCe1 and vinculin is indicated as merge and with yellow staining. DICM, differential interference contrast microscopy. Bar = 30 μm.

experiments, using sera incubated with the immunogenic fusion proteins (containing the sequences of the EC3 or EC4 loops). The effects of both antibodies on NBCe1 activity were evaluated in the presence of the respective fusion proteins (Figure 5C). Under these conditions, the inhibitory action of a-L3 and the stimulatory effect of a-L4 were blocked, indicating that removal of anti-EC antibodies removed the action from the sera.

Dose-response curves of the effect of a-L3 (Figure 6A) and a-L4 (Figure 6B) on the NBCe1 transport activity showed that the serum concentration containing a-L3 or a-L4 used in the experiments of Figure 5 (1/500) produced maximal responses on NBCe1 activity.

Opposing functional effects of a-L3 and a-L4 on NBCe1 activity evaluated with the ammonium pulse

We have recently studied the participation of NBC in pH_i recovery, after acidification induced by the ammonium pulse in cat isolated ventricular myocytes (De Giusti *et al.*, 2009). In order to assess the differential activity of NBCe1 in this pH_i recovery, we pre-incubated the cells 10 min before an ammonium pulse with the a-L3 or a-L4 (1/500 dilutions). These experiments were performed in the continuous presence of 10 μmol·L^{-1} HOE642, in order to eliminate the participation of the NHE during the recovery of pH_i. The a-L3 decreased and the a-L4 increased the NBC-mediated J_H in comparison with N-I serum (control), indicating that NBCe1 contributed to pH_i recovery from acidosis in cat ventricular myocytes (Figure 7). The effects of a-L3 and a-L4 were also evaluated in the ammonium pulses in the absence of HCO_3^- and HOE642 (Supporting Information Figure S4). Under these conditions, no effects were observed, indicating that these sera exert their effects on a bicarbonate-dependent alkalinizing mechanism without affecting the activity of NHE.

The inhibitory effect of a-L3 is supported by previous results from Khandoudi *et al.* (2001), who also found that a polyclonal antibody directed against a short sequence of NBCe1 EC3 region slowed pH_i recovery from acidosis in rat ventricular myocytes (Khandoudi *et al.*, 2001). The previous study did not, however, examine the rat cardiac NBCe1 activity in isolation. Nevertheless, taken together, these experiments suggest that these antibodies might represent a useful tool to functionally discriminate the transport activity of NBC isoforms.

Opposing functional effects of a-L3 and a-L4 on NBCe1 activity evaluated with perforated patch

We have recently demonstrated that eNBC contributes to the normal configuration of the AP waveform of cat ventricular myocytes (Villa-Abrille *et al.*, 2007). We reported that chang-

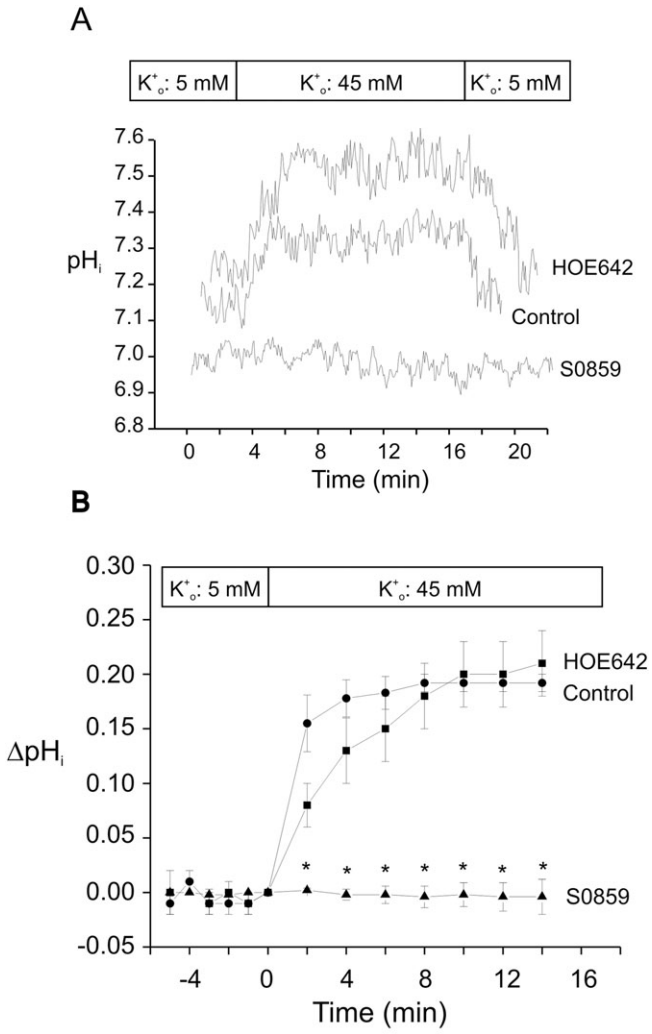

Figure 4

Isolation of the eNBC activity with the potassium pulse. (A) Representative traces of pH_i recorded from myocytes exposed to the potassium pulse in control and in the presence of the NHE blocker HOE642 (10 μmol·L^{-1}) or the NBC blocker S0859 (10 μmol·L^{-1}). (B) Average data of pH_i alkalinization induced by the high potassium-induced depolarization of membrane potential in control ($n = 6$) and in the presence of the inhibitor of NHE, HOE642 (10 μmol·L^{-1}, $n = 7$) or the inhibitor of NBC, S0859 (10 μmol·L^{-1}, $n = 5$). Data are expressed as increase of pH_i units in comparison with the zero time point in high potassium solution. *$P < 0.05$ significantly different from control and HOE642.

ing the superfusate bathing myocytes from a HCO_3^--free (HEPES buffer) to a HCO_3^--containing solution at constant pH_o, induces 15–25% shortening of AP duration (APD), consistent with the influx of HCO_3^- into the cell. These changes were sensitive to the extracellular concentration of HCO_3^- and were blunted by anionic blockade or by sodium deprivation (Villa-Abrille *et al.*, 2007). In the present work, we evaluated the participation of NBCe1 in this eNBC-mediated APD shortening. We examined APs recorded in HCO_3^--containing solution at constant pH_o, before and after exposing the myocytes to N-I serum, serum containing a-L3 or serum contain-

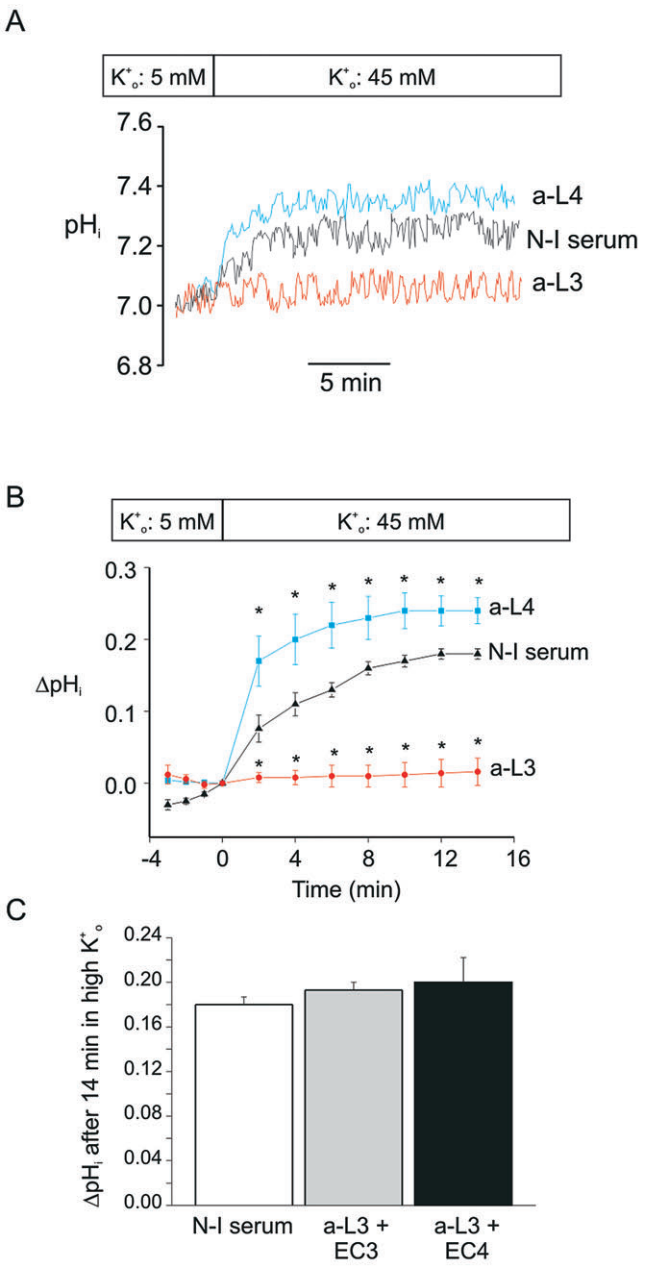

Figure 5

Effects of a-L3 and a-L4 on NBCe1 activity evaluated with the potassium pulse. (A) Representative traces of pH_i recorded from myocytes exposed to the potassium pulse in the presence of the N-I serum (1:500), a-L3 (1:500) or a-L4 (1:500). (B) Average time course of the increase in pH_i elicited by 14 min exposure to high potassium in the absence (N-I serum, 1:500, $n = 5$) and presence of a-L3 (1:500, $n = 5$) or a-L4 (1:500, $n = 6$). Data are expressed as increase of pH_i units in comparison with the zero time point in high potassium solution. *$P < 0.05$ significantly different from N-I serum. (C) Average data obtained after 14 min of myocyte exposure to N-I serum ($n = 5$) or serum with a-L3 ($n = 4$) or a-L4 ($n = 4$) co-incubated with the fusion proteins (1 μg ml^{-1}, 1 h co-incubation), containing the sequences of the EC3 or EC4 loops.

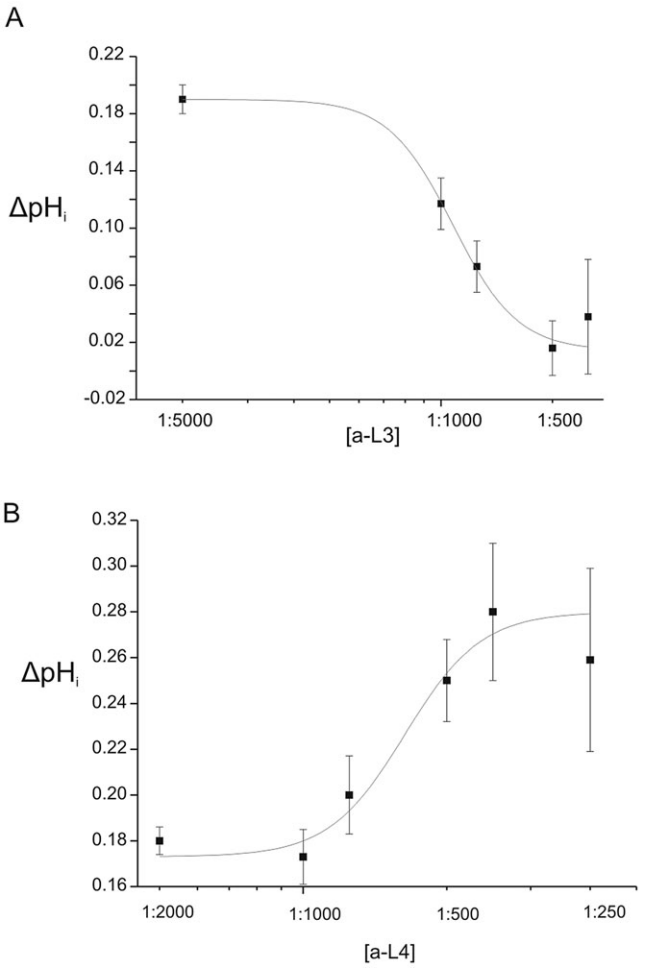

Figure 6

Dose-response curve of the effect of a-L3 and a-L4 on NBCe1 transport activity. (A) Dose-response effect of a-L3 on NBC1 activity at the following concentrations of the serum containing the antibodies (dilution v/v): 1:5000 (n = 5), 1:1000 (n = 4), 1:800 (n = 5), 1:500 (n = 5), 1:400 (n = 4). Values were obtained at 14 min following application of the K^+ pulse. (B) Dose-response effect of a-L4 on NBCe1 activity at the following concentrations of the serum containing the antibodies (dilution v/v): 1:2000 (n = 5), 1:1000 (n = 4), 1:800 (n = 4), 1:500 (n = 7), 1:400 (n = 4), 1:250 (n = 4). Values were obtained at 14 min following application of the K^+ pulse. As stated in the text (not shown in the present Figure), the concentration of the N-I serum used was 1:500 and the value of ΔpH_i obtained was 0.18 ± 0.01 (n = 5).

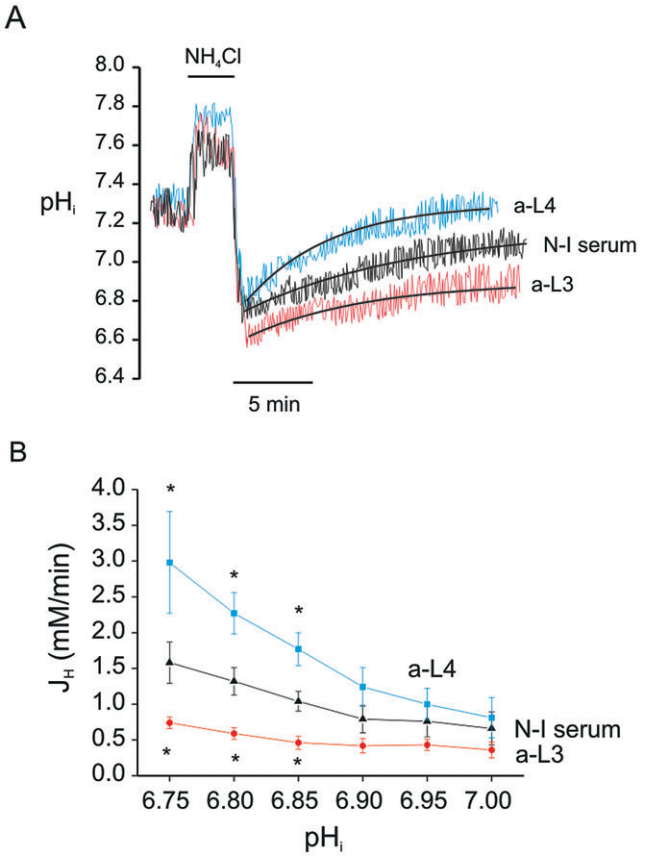

Figure 7

Effects of a-L3 and a-L4 on NBCe1 activity evaluated with the ammonium pulse. (A) Superimposed representative recordings of pH_i obtained from myocytes exposed to an ammonium pulse in the absence (N-I serum) or the presence of a-L3 (1:500) or a-L4 (1:500). The experiments were performed in the continuous presence of HOE642 in order to block NHE-1 activity. (B) Average J_H calculated at different pH_i values in the absence (serum sham, 1:500, n = 5) and presence of a-L3 (1:500, n = 5) or a-L4 (1:500, n = 6). *P < 0.05 significantly different from N-I serum.

ing a-L4 (Figure 8A). Whereas N-I serum had no effect on APD, a-L3 prolonged and a-L4 shortened APD, as expected from the effects of these antibodies in the experiments of pH_i measurements. Analysis of averaged data showed that serum containing a-L3 induced an APD lengthening (measured at 90% of repolarization time, APD_{90}) of approximately 17%, consistent with blockade of NBCe1. In contrast, serum containing a-L4 induced an average APD_{90} shortening of almost 20%, according to stimulation of NBCe1 (Figure 8B). Parallel experiments performed in the absence of HCO_3^- (HEPES-buffered solution) demonstrated that the antibodies did not exert significant effects (Supporting Information Figure S5), suggesting that they do not affect currents other than that generated by NBCe1. During the recordings of APs in the presence of bicarbonate, it is possible that the selective action of the antibodies on NBCe1 could affect pH_i and therefore modify channel activity and APD. However, this possibility seems very unlikely as the changes in pH_i that would be induced by the activation or inhibition of NBCe1 at basal pH_i should not be able to induce significant effects on ionic currents. Please note that these recordings were performed in the presence of an active NHE-1. It has been previously demonstrated that exclusive inhibition of NHE-1 or NBC does not affect basal pH_i, as the sole participation of each of these transporters should be sufficient to maintain basal pH_i at a relative constant level (Vandenberg *et al.*, 1993). Furthermore, we have previously demonstrated that at basal pH_i, the activation of NBCe1 generates a 4-acetamido-4-isothiocyanatostilbene-2,2′-disulphonic acid (SITS)-sensitive anionic current in the absence of changes in pH_i (Aiello *et al.*,

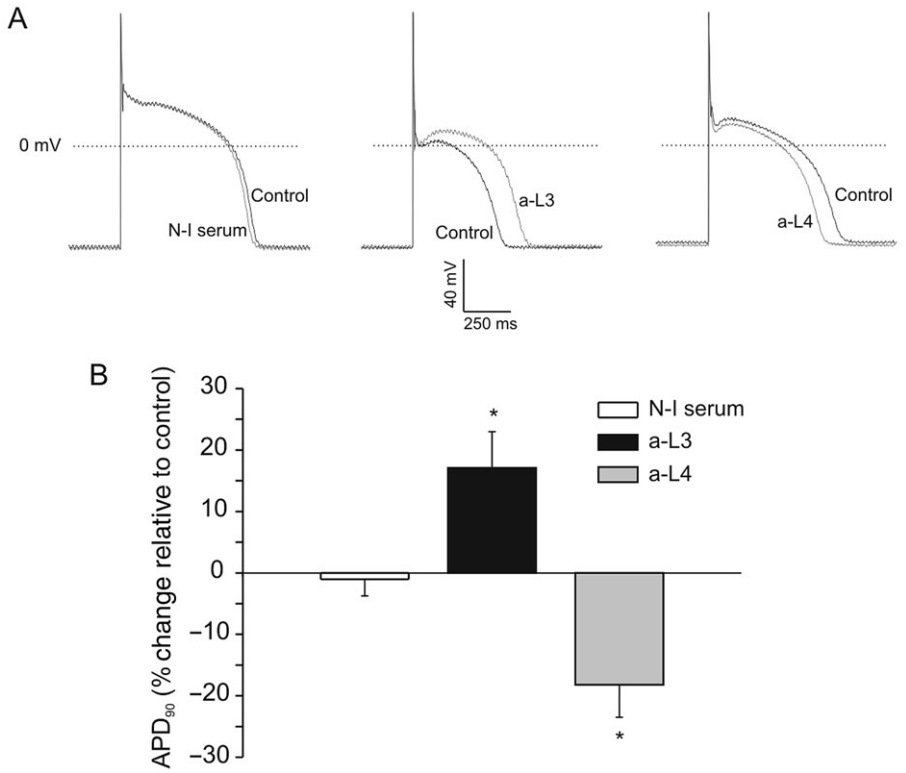

Figure 8

Effects of a-L3 and a-L4 on NBCe1 activity, evaluated with perforated patches of cat isolated cardiomyocytes. (A) representative traces of action potentials, recorded in HCO_3^- containing solution at constant pH_o, before and after exposing the myocytes during 10 min to N-I serum, serum containing a-L3 or serum containing a-L4. (B) Average effects of N-I serum (1:500, $n = 6$), a-L3 (1:500, $n = 6$) and a-L4 (1:500, $n = 6$) on APD_{90} expressed as % change relative to the control values. The data were obtained 10 min after the treatment with the respective sera. *$P < 0.05$ significantly different from N-I serum.

1998; Villa-Abrille *et al.*, 2007). Together, these electrophysiological data confirm the participation of eNBC in the normal configuration of the AP waveform of cat ventricular myocytes, as suggested in our previous work (Villa-Abrille *et al.*, 2007), and position the current generated by NBCe1 as the main, and may be the only, component of the total anionic current carried by eNBC in these cells.

Discussion and conclusions

Polyclonal antibodies are useful to detect proteins with low expression levels as more antibodies can bind to a single protein molecule, thus enhancing the detection signal. Polyclonal antibodies raised against recombinant peptides corresponding to amino acid sequences of a protein have proven to be useful not only in understanding the structure, and molecular forms of AE1 and AE3 Cl^-/HCO_3^- exchanger and NBCe1, but also for studying functional aspects of the transporters (Puceat *et al.*, 1998; Khandoudi *et al.*, 2001; Chiappe de Cingolani *et al.*, 2006). Here we generated polyclonal antibodies against the putative third, EC3, and fourth, EC4, extracellular loops of rat NBCe1. Target antigenic sequences selected were predicted as flexible regions without β-sheet or α-helix secondary structures, with at least 24% of one of the following amino acids: R, N, Q, K, D or E, and presence of Y and/or P amino acids conferring structural motif to the antigen. The antigen-binding site of a typical antibody is a cleft formed by folded variable heavy and variable light regions. It can accommodate approximately four to seven amino acids or sugar residues and such amino acid sequence conformation is not preserved comparing the EC3 and EC4 of NBCe1 and other bicarbonate transporters (Figure 1A). Nevertheless, we were also able to confirm that a-L3 or a-L4 do not recognize other NBC isoforms (NBCe2 or NBCn1, Figure 2A).

Antibodies were able to detect NBCe1 on immunoblots using cat cardiac ventricles or lysates of NBCe1-transfected HEK293 cells (Figure 1). The antisera also detected the NBCe1 protein in immunoprecipitation experiments conducted in cat and rat myocardium (not shown), demonstrating the role of the antibodies when the native protein conformation (denaturing conditions) was preserved. In addition, a-L3 and a-L4 were helpful in studying the localization of NBCe1 in isolated cardiomyocytes by immunocytochemistry, proving the ability of the antibodies to recognize folded tertiary structures of the NBCe1 protein (Figure 3).

The NBC and the NHE are the main acid-extruders in cardiac myocytes and contribute equally to the regulation of basal pH_i (Lagadic-Gossmann *et al.*, 1992; Camilion de

Hurtado *et al.*, 1995; Le Prigent *et al.*, 1997; Vaughan-Jones *et al.*, 2006; 2009). The employment of several selective pharmacological blockers of the NHE (Bountra and Vaughan-Jones, 1989; Harrison *et al.*, 1992; Scholz *et al.*, 1993; Knight *et al.*, 2001) proved helpful in documenting the participation of this transporter in cardiac physiology. Moreover, these compounds were beneficial in different pathophysiological settings, including ischaemia/reperfusion (Scholz *et al.*, 1995; Clements-Jewery *et al.*, 2004) and myocardial hypertrophy (Cingolani and Ennis, 2007). In contrast, the lack of specific NBC blockers has made it difficult to investigate the contribution of this bicarbonate transporter to cardiac physiology and pathophysiology. Thus, it is important to mention the availability of a new compound, S0859, which selectively blocks NBC (Ch'en *et al.*, 2008). This compound, however, inhibited all cardiac NBC activity, making it a broad-spectrum NBC inhibitor that is not NBC isoform selective (Ch'en *et al.*, 2008), as also recently demonstrated in cat ventricular myocytes (De Giusti *et al.*, 2009). Therefore, the ability of a-L3 to block NBCe1 selectively, opens new and valuable paths to dissect the specific NBCe1 activity.

The data presented in this work demonstrated that the predominant electrogenic alkalinizing mechanism in cat ventricular myocytes was NBCe1, suggesting that either NBCe2 was not expressed at all or it was expressed, but barely detectable in the potassium pulse. Although data supporting expression of NBCe2 in the heart have been previously reported (Pushkin *et al.*, 2000; Virkki *et al.*, 2002), the presence of this NBC isoform in myocardium has been questioned (Yamamoto *et al.*, 2007). It is also possible, however, that NBCe2 may be present in heart cells other than myocytes, as it was suggested for NBCn1 in rat and mouse myocardium (Damkier *et al.*, 2006; Boedtkjer *et al.*, 2008).

We have previously demonstrated in cat ventricular myocytes that the bicarbonate-mediated and cariporide insensitive pH_i recovery from acidosis was totally blocked by SITS or S0859, indicating that it is mediated by NBC (De Giusti *et al.*, 2009). As a-L3 reduced J_H by approximately 50%, we speculate that in cat ventricular myocytes, both isoforms, electroneutral NBCn1 and electrogenic NBCe1, are functionally active, exhibiting equivalent participation in the regulation of pH_i. However, we cannot rule out the possibility that the activity of NBCe2 could be barely detectable with the potassium pulse but could be more important during the recovery from acidosis in the ammonium pulse. Thus, we cannot completely exclude the participation of NBCe2 in the remaining recovery from the acid load in the presence of a-L3.

If we consider that the main NBC isoforms of cardiac myocytes are NBCe1 and NBCn1 and because NBCe1 mediates the co-influx of 1 Na^+ and two HCO_3^- ions, this transporter would carry one third of the total Na^+ loading induced by the NBC-mediated HCO_3^- transport. Although intracellular Na^+ (Na^+_i) measurements are needed to confirm this issue, it is feasible to anticipate that the 'Na^+ sparing' HCO_3^- flux induced by NBCe1 would lead to less deleterious effects on cardiomyocyte function in comparison with NBCn1 in some enhanced Na^+_i scenarios, like that produced during pH_i recovery from acidosis upon reperfusion after ischaemia. Selective blockade of NBCe1 during reperfusion after low-flow ischaemia, however, induced almost complete recovery of contractile parameters, in rat isolated perfused hearts (Khandoudi *et al.*, 2001). Further research is needed to give clearer insights into this matter.

The idea that functional antibodies can be used as pharmacological tools is not new (Haber, 1992). Antibodies that produce loss of function (Puceat *et al.*, 1998; Khandoudi *et al.*, 2001; Chiappe de Cingolani *et al.*, 2006) or gain of function (Borda *et al.*, 1984; Magnusson *et al.*, 1994) of targeted cardiac proteins have been previously reported. As far as we can find, this is the first study to identify antibodies directed against different portions of the same cardiac protein that evoke opposite effects on the function of the antigen. The investigation of the possible mechanisms involved in the inhibition and stimulation of NBCe1 by a-L3 and a-L4, respectively, did not constitute an objective of the present work. On the basis of the modular structure of sodium-coupled bicarbonate transporters (Boron *et al.*, 2009), some speculations about the mechanistic action of these antibodies can be made. It is well accepted that the anion blockers, stilbene-derived SITS and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, covalently couple to the first lysine in a KXXK motif at the extracellular end of trans-membrane segment 5, which is sequentially adjacent to EC3 of most bicarbonate transporters, including all NBC isoforms (Boron *et al.*, 2009). Previous data (Khandoudi *et al.*, 2001) and the present work show that antibodies produced against EC3 block NBCe1 activity, which lends support to the hypothesis that these antibodies interfere with anion transport in a similar fashion to the stilbene derivatives. Furthermore, a similar blocking effect of the activity of the AE3 induced by a functional antibody directed against the EC3 (but not against the EC4) has been previously reported (Chiappe de Cingolani *et al.*, 2006). Conversely, conformational changes of the transporter induced by a-L4 could explain the stimulatory effect of this antibody on NBCe1 activity. These changes could favour dimerization of the protein (Kao *et al.*, 2008) or facilitate the interaction between NBCe1 and the carbonic anhydrase (CA) (Alvarez *et al.*, 2003). However, at least for the interaction with CAIV, the latter possibility seems unlikely because the binding site for this CA is localized in the EC4 (Alvarez *et al.*, 2003).

Evidence for a causal relationship between NBC and cardiac pathophysiology are still lacking. NBC is, however, activated during reperfusion after ischaemia (Schafer *et al.*, 2000; Doggrell and Hancox, 2003; van Borren *et al.*, 2004; Ten Hove *et al.*, 2005) and the damage produced by this pathology can be attenuated by blockade of this transporter (Khandoudi *et al.*, 2001), suggesting that NBCe1 activity could be harmful in this setting. In addition, up-regulation of the expression of NBC in hearts of patients with heart failure (Khandoudi *et al.*, 2001) and in hearts of infarcted animals (Sandmann *et al.*, 2001) has been also reported. More recently, expression of NBCe1 and NBCn1 in hypertrophic rat hearts was reported (Yamamoto *et al.*, 2007). As corroborated herein, NBCe1 produces an anionic repolarizing current, which would attenuate the characteristic prolongation of APD present in cardiac hypertrophy (Carmeliet, 2006), opening the question of whether NBCe1 up-regulation would be compensatory and beneficial, or deleterious. Thus, the selective opposing effects of a-L3 and a-L4 on NBCe1 function may provide insight into the possibility that NBCe1 activity could be a 'friend or foe' depending on the cardiac pathological state studied.

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Conflicts of interest

None.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Measurement of the βi. βi of the myocytes was measured by exposing cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. pH_i was allowed to stabilize in Na^+-free solution before application of NH_4Cl. βi was calculated from the equation $\beta i = \Delta[NH_4^+]i/\Delta pH_i$ and referred to the mid-point values of the measured changes in

pH_i. βi at different levels of pH_i was estimated from the least squares regression lines βi versus pH_i plots. Na^+-free HEPES bathing solution (in mmol·L^{-1}): HEPES 25, KCl 4.66, $MgSO_4$ 1.05, $CaCl_2$ 1.35, N-methyl-D-glucamine 148, glucose 11 and NH_4Cl 0, 3, 5, 9, 12, 15 or 20, pH adjusted to 7.4 with HCl. The figure shows the βi versus pH_i plot and the regression line used to fit it (n = 8).

Figure S2 Specificity of the potassium pulse to evaluate eNBC activity. Average data of the increase in pH_i obtained after 14 min of exposure of the myoctes to high K^+ in the absence of bicarbonate (0 HCO_3^-, n = 6) or sodium (0 Na^+, n = 4).* indicates $P < 0.05$ versus control.

Figure S3 Specific action of a-L4 on NBCe1 evaluated in the potassium pulse. Average data of the increase in pH_i obtained after 14 min of exposure of the myocytes to high K^+ in the presence of the N-I serum (n = 5) or in the continuous presence of a-L4 co-incubated with S0859 (n = 4) or in the absence of bicarbonate (n = 3) or sodium (n = 4). * indicates $P < 0.05$ versus N-I serum.

Figure S4 Specific action of a-L3 and a-L4 evaluated in the ammonium pulse in 0 HCO_3^-. Average J_H calculated at pH_i 6.8 in the absence (N-I serum, 1:500, n = 4) and presence of a-L3 (1:500, n = 3) or a-L4 (1:500, n = 4). These experiments were performed in the absence of bicarbonate (HEPES-buffered solution) and in the absence of the NHE-1 inhibitor, HOE642. Under these conditions, the only active alkalinizing mechanism is the NHE-1.

Figure S5 No effects of a-L3 and a-L4 on APD in the absence of HCO_3^-. Representative traces of action potentials recorded in extracellular solution without HCO_3^- (HEPES-buffered solution). The traces were recorded before and after 10 min of exposing the myocytes to serum, containing a-L3 (1:500) or serum containing a-L4 (1:500). As observed, no effects of the antibodies were observed under these conditions. On average, the percentage effects relative to control on APD_{50} were –0.51 ± 1.24% (n = 5) and 0.28 ± 1.51% (n = 5) with a-L3 and a-L4 respectively. The percentage effects relative to control on APD_{90} were –0.22 ± 0.85% (n = 5) and –0.32 ± 1.51% (n = 5) with a-L3 and a-L4 respectively.

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