Volume 289, number 2, 221–223 FEBS 10142 September 1991

ADONIS 001457939100854D

Detection of Na^+/H^+ exchanger mRNA in human neutrophils and lymphocytes using the polymerase chain reaction

Beate Quednau[1], Dieter Rosskopf[1], Hartmut Weiler-Güttler[2] and Winfried Siffert[1]

[1]*Max-Planck-Institut für Biophysik, Kennedyallee 70, D-6000 Frankfurt/Main 70, Germany* and [2]*Institut für Biochemie der Technischen Hochschule Darmstadt, D-6100 Darmstadt, Germany*

Received 21 May 1991; revised version received 9 July 1991

Using the reverse polymerase chain reaction (RT-PCR), we have examined the expression of Na^+/H^+ exchanger mRNA in human buffy coat preparations, lymphocytes and neutrophils. Total RNA from all cell types was reverse transcribed specifically and then amplified by PCR. The identity of the PCR products was confirmed by restriction enzyme analysis and hybridization with a specific oligonucleotide probe. The detection of low abundance Na^+/H^+ antiporter specific transcripts by RT-PCR in different human blood cells ex vivo should facilitate future studies on regulatory and pathophysiological aspects of Na^+/H^+ exchanger mRNA expression in human cells and tissue samples.

Na^+/H^+ exchange; Neutrophil; Lymphocyte; Polymerase chain reaction; Hypertension

1. INTRODUCTION

Regulation of intracellular pH (pH_i) in vertebrate cells is ensured by a specific ion transport system termed the Na^+/H^+ exchanger which provides an electroneutral exchange of intracellular H^+ for extracellular Na^+. This ion transport serves a variety of cellular functions, e.g. pH_i - homeostasis, control of cell volume and regulation of cell proliferation [1]. The activity of the Na^+/H^+ exchanger is enhanced after cell stimulation, e.g. by growth factors most likely by phosphorylation of a regulatory site in a protein kinase C-dependent reaction (for reviews see e.g. [1,2).

Although the human amiloride-sensitive Na^+/H^+ exchanger has recently been cloned from transformed mouse fibroblasts [3], the actual expression of Na^+/H^+ antiporter-specific mRNA in human cells and tissues remained to be demonstrated.

We investigated the functional presence of Na^+/H^+ exchange in human lymphocytes and neutrophils and the expression of Na^+/H^+ antiporter-specific mRNA in these blood cells by use of the reverse transcription polymerase chain reaction (RT-PCR).

2. MATERIALS AND METHODS

2.1. *Preparation of blood cells*

Blood (100 ml) was obtained from human volunteers and anticoagulated with 20% (v/v) acid–citrate–dextrose (75 mM sodium citrate, 38 mM citric acid and 138 mM glucose). Neutrophils were isolated from whole blood after dextran sedimentation and hypotonic lysis using standard methods [5]. Lymphocytes were isolated by centrifugation through Ficoll Hypaque (Pharmacia LKB, Uppsala, Sweden) [6]. Buffy coat preparations from human blood concentrates were kindly provided by Dr Wolter, German Red Cross Blood Transfusion Center, Hagen, Germany.

Correspondence address: W. Siffert, Max-Planck-Institut für Biophysik, Kennedyallee 70, D-6000 Frankfurt/Main 70, Germany. Fax: (49) (69) 6303244.

2.2. *Measurement of Na^+/H^+ exchange*

Cells were suspended in HEPES buffer (140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES (Na^+-free), 5 mM glucose, pH 7.4, and incubated with 20 μM of the pH-sensitive fluorescent dye 2',7'-bis(carboxyethyl)-5,6-carboxy-fluorescein acetoxymethylester (BCECF-AM; Molecular Probes, Oregon, USA) for 30 min at 37°C. BCECF-loaded cells were transferred to a cuvette containing 2 ml of HEPES buffer to yield a final concentration of 1×10^7 cells/ml and prewarmed to 37°C. The fluorescence of BCECF was recorded in a KONTRON SFM 25 spectrofluorimeter (Kontron, Düsseldorf, Germany) equipped with a thermostatted cuvette holder as previously described [7]. Na^+/H^+ exchange was activated by the addition of Na^+-propionate solution, pH 7.4, to a final concentration of 30 mM.

2.3. *Preparation of RNA*

Total RNA was isolated from buffy coat preparations and granulocytes using the guanidinium thiocyanate method including purification of the RNA by CsCl-gradient centrifugation [8]. Total RNA from lymphocytes was isolated using the method of Chomczynski and Sacchi [9].

2.4. *Synthesis of cDNA*

One microgram of total RNA was reverse transcribed specifically at 37°C for 60 min with 200 U of Moloney urine leukemia virus (M-MLV) reverse transcriptase (BRL, Gaithersburg, USA) and the following reagents (in a total volume of 100 μl): 1 × reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM DTT, 1.5 mM $MgCl_2$); 10 pmol primer I; 25 U RNase inhibitor (Boehringer, Mannheim, Germany); 1 mM dATP, dGTP, dCTP, dTTP, respectively. The reaction was stopped by heating the solution to 95°C for 5 min. The tubes were then cooled to 4°C and stored on ice or frozen until used.

2.5. *Oligonucleotides*

Oligonucleotide primers (see Table I) were synthesized by the phosphoramidite solid-phase method on an Applied Biosystems Model 380A synthesizer. Primer I (used for cDNA synthesis) is complementary to bases 384 to 368 of the human antiporter cDNA sequence

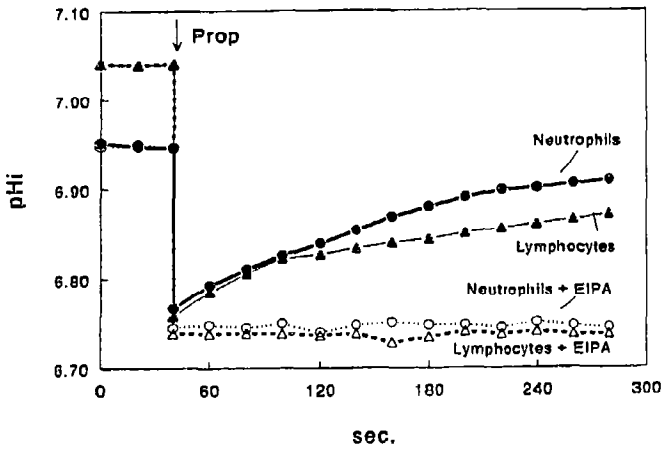

Fig. 1. Representative curves showing pH_i recovery from artificial cytosolic acidification. (A) human lymphocytes; (B) human neutrophils. Na^+-propionate (Prop), 30 mM, was added at the arrows. The displayed curves are representative of 8 experiments each.

[3]. Primer II is complementary to bases 351 to 334 of the human cDNA and primer III is identical to bases −3 to +21. Primer III additionally carries an artificial *Bam*HI site and a stuffer comprising 4 nucleotides. The sequence flanked by primers II and III contains a single *Nsp*V site (TT/CGAA). Oligonucleotide IV was used for Southern blot hybridization of the PCR products. It is complementary to bases 145 to 128 of the human cDNA sequence.

2.6. *Polymerase chain reaction (PCR)*

PCR was carried out as described by Kawasaki and Wang [10] and Doherty et al. [11] with minor modifications. One-tenth volume of the cDNA reaction mixture was amplified in a total volume of 50 μl containing 1 × PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.1 mg/ml BSA), 200 μM dATP, dCTP, dGTP, dTTP, respectively, 1 mM DTT, 10 pmol primer II, 10 pmol primer III. Samples were heated first to 94°C for 5 min before 2.5 U of Taq DNA-Polymerase (Perkin- Elmer-Cetus) were added and the reaction mixture was overlaid with 40 μl mineral oil (Sigma, St. Louis, USA). PCR was carried out in a programmable heater (Biomed, Obertheres, Germany). Amplification was performed for 40 cycles, each cycle consisting of denaturation at 94°C (30 s), annealing at 60°C (1 min), and elongation at 72°C (90 s). All samples were kept on ice or frozen until analyzed.

A blank was prepared using all of the reagents including 200 ng of RNA and the Na^+/H^+ antiporter PCR primers II and III.

2.7. *Restriction enzyme analysis*

8 μl of each PCR reaction mixture was ethanol-precipitated, redissolved in 10 μl water and incubated with 10 U of the restriction enzyme *Nsp*V and the corresponding 10× buffer (Biozym, Hameln, Germany) for 1 h at 50°C (25 μl, final volume) before another 10 U of the enzyme were added for one more hour. The samples were separated on a 1 mm 8% polyacrylamide gel using pBR 322/*Hae*III fragments as size standards.

2.8. *Southern blot hybridization*

7 μl of each PCR reaction was separated on a 2% agarose gel and the DNA was blotted onto GeneScreen membrane (DuPont NEN, Boston, USA). Hybridization occurred at 51°C for 16 h in a solution containing 6× SSC, 3× Denhardt's solution, 1.0% SDS, 100 μg/ml denatured herring sperm DNA with 5×10^5 cpm/ml of the labeled oligonucleotide probe IV. 5'-Phosphorylation of the probe was carried out using [t^{32}P]ATP and T4 polynucleotide kinase (BRL, Gaithersburg, USA). Filters were washed four times in 6× SSC, 0.1% SDS at 51°C for 10 min, one time in 2× SSC, 0.1% SDS at 51°C for 5 min, and exposed to Kodak X-AR film for 16 h.

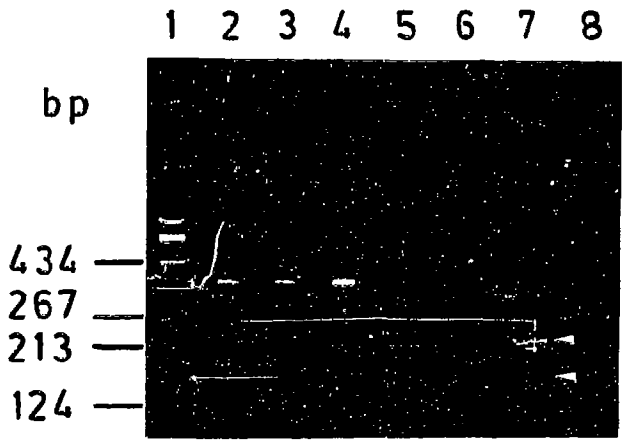

Fig. 2. Detection of Na^+/H^+ exchanger-specific mRNA in human blood cells by RT-PCR and restriction fragment analysis of the PCR products. Lane 1, size standard; lane 2, buffy coat; lane 3, lymphocytes; lane 4, neutrophils. Lanes 5–7 display the PCR products after digestion with *Nsp*V. Lane 5, buffy coat; lane 6, lymphocytes; lane 7, neutrophils. Lane 8, blank. Samples were separated on a 1 mm 8% polyacrylamide gel using pBR 322/*Hae*III fragments as size standard.

3. RESULTS AND DISCUSSION

The recovery of pH_i from acidification in neutrophils and lymphocytes is depicted in Fig. 1. Both cell types acidified upon the addition of propionate and, thereafter, pH_i gradually returned to its initial value. This response was inhibited both after removal of extracellular sodium (not shown) or in the presence of 20 μM EIPA which confirms the presence of a Na^+/H^+ exchanger in neutrophils and lymphocytes (for review see [4]).

Next we examined the expression of the Na^+/H^+ antiporter mRNA in human blood cells. Total RNA from buffy coat, lymphocytes, and neutrophils was reverse-transcribed specifically and the resulting cDNA was subjected to amplification by PCR. The obtained PCR products are shown in Fig. 2. After amplification a single major DNA band was obtained in each lane. The size of these bands corresponds to the predicted length of 364 bp as deduced from a suitable standard size marker. The identity of the PCR products as amplified human Na^+/H^+ exchanger-specific cDNA was confirmed first by restriction enzyme analysis using *Nsp*V (see section 2), which yielded two fragments of the expected

Table I

Oligonucleotides corresponding to the human Na^+/H^+ exchanger cDNA sequence

Oligonucleotide	Sequence (5'→3')
Primer 1 (cDNA)	GATGCTTGAGATAGTGG
Primer 2 (PCR)	TATCTTCATGAGGCAGGC
Primer 3 (PCR)	GATCGGATCCAC-CATGGTTCTGCGGTCTGGCATC
Oligo 4 (hybridization)	AGCTTCGAATGGTGCTGG

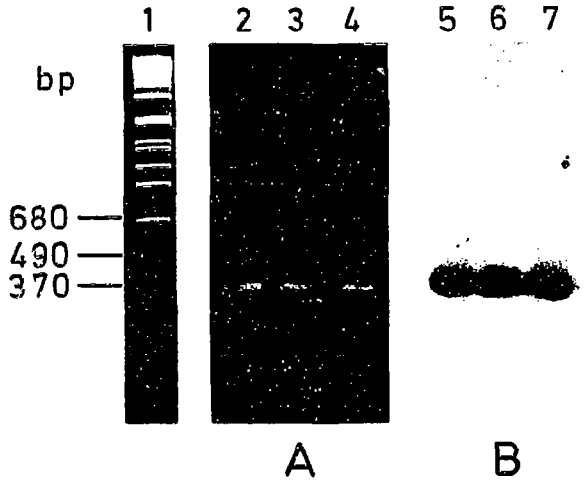

Fig. 3. Southern blot hybridization of RT-PCR products with a Na^+/H^+ antiporter-specific oligonucleotide probe. (A) PCR products after amplification of cDNAs; (B) autoradiograph of a corresponding Southern blot after hybridization with oligonucleotide 4 (cf. Table I). Lane 1, size standards; lanes 2 and 5, buffy coat; lanes 3 and 6, neutrophils; lanes 4 and 7, lymphocytes. The PCR products were separated on an ethidium bromide-stained 2% agarose gel using SPP1/*Eco*RI fragments as size standard.

size (213 and 151 bp). The weak DNA bands on the same level as the unrestricted products result from a non-complete digestion of the cDNA. Subsequently, the PCR products were separated on a 2% agarose gel and analyzed by Southern blot hybridization (Fig. 3). The three DNA bands formed during PCR strongly hybridized with the Na^+/H^+ exchanger-specific oligonucleotide probe. These results, the generation of PCR products of exactly the predicted size, the presence of a single *Nsp*V site, together with the hybridization results, positively identify the PCR products as amplified cDNA segments encoding the human Na^+/H^+ exchanger.

We have described here a PCR method for studying the expression of Na^+/H^+ exchanger-specific mRNA in human blood cells. The amount of total RNA that can be prepared from resting human blood cells of a single donor is far below the quantity required for conventional methods of mRNA detection, e.g. Northern blotting. Hence, for studies on the expression of Na^+/H^+ antiporter mRNA in human cells and tissue samples, a highly sensitive method is essential.

The present report should also facilitate the development of a quantitative PCR assay for the detection of Na^+/H^+ exchanger mRNA in blood cells from subjects suffering from essential hypertension. These patients display an enhanced activity of Na^+/H^+ exchange in e.g. platelets and leukocytes [12,13] which may result from an increased number of active transport proteins.

Acknowledgements: We are indebted to Dr Wolter, Blutspendedienst Nordrhein-Westfalen des Deutschen Roten Kreuzes, Hagen, for supplying us with buffy coat preparations. This study was supported by a grant from the Deutsche Forschungsgemeinschaft and by MSD Sharp & Dohme, Munich, Germany.

REFERENCES

[1] Frelin, C., Vigne, P., Ladoux, A. and Lazdunski, M. (1988) Eur. J. Biochem. 174, 3–14.
[2] Sardet, C., Counillon, L., Franchi, A. and Pousségur, J. (1990) Science 247, 723–726.
[3] Sardet, C., Franchi, A. and Pouysségur, C. (1989) Cell 56, 271–280.
[4] Grinstein, S. (ed.) (1988) Na^+/H^+ exchange, CRC Press, Boca Raton, FL, USA.
[5] Chanarin, I. (ed.) (1989) Laboratory Hematology, pp. 177–179, Churchill-Livingstone, Edinburgh-London.
[6] Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, suppl. 21, 77.
[7] Siffert, W., Siffert, G., Scheid, P. and Akkerman, J.W.N. (1989) Biochem. J. 258, 521–527.
[8] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Ruttler, W.J. (1979) Biochemistry 18, 5294–5299.
[9] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[10] Kawasaki, E.S. and Wang, A.M. (1989) in: PCR Technology (Erlich, H.A., ed.), pp. 89–99, Stockton Press, New York.
[11] Doherty, P.J., Huesca-Contreras, M., Dosch, H.M. and Pan, S. (1989) Anal. Biochem. 177, 7–10.
[12] Rosskopf, D., Morgenstern, E., Scholz, W., Osswald, U. and Siffert, W. (1991) J. Hypertension 9, 231–238.
[13] Dudley, C.R.K., Taylor, D.J., Ng, L.L., Kemp, G.J., Ratcliffe, P.J., Radda, G.K. and Ledingham, J.G.G. (1990) J. Hypertension 79, 491–497.