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Cloning of two isoforms of the rat brain Na^+–Ca^{2+} exchanger gene and their functional expression in HeLa cells*

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Two functional isoforms of the rat brain Na^+–Ca^{2+} exchanger were isolated from a λZAP hippocampus cDNA library. The open reading frame of clone RBE-1 codes for a protein 935 amino acids long, and that of clone RBE-2 codes for a protein 958 amino acids long. Expression in HeLa cells of Na^+ gradient dependent Ca^{2+} transport activity was determined following transfection of the cells with either RBE-1 or RBE-2. Both clones expressed proteins that exchange Na^+ with Ca^{2+} in an electrogenic manner and none of them exhibited a dependency of the antiport on K^+, since they transported Ca^{2+} in an Na^+ gradient dependent manner in external choline chloride as well.

Na^+–Ca^{2+} exchange; Calcium regulation; Brain; Cloning; Expression; HeLa cell

1. INTRODUCTION

Na^+ gradient dependent Ca^{2+} fluxes are a major form of intracellular Ca^{2+} ion regulation in all excitable and many non-excitable cells [1]. The extent and direction of these fluxes depends primarily on the relative Na^+ and Ca^{2+} gradients across the membrane, the membrane potential and the stoichiometry of the process [2,3]. In addition, Na^+–Ca^{2+} exchange is affected by the variable affinity of the exchanger protein to Ca^{2+} under different physiological conditions [4,5], its modulation by ATP [6,7], calmodulin and/or phosphorylation [8]. Experiments designed to evaluate the role of these secondary parameters in regulating Na^+–Ca^{2+} exchange, resulted in unexplicably variable and often controversial results [5,9].

In this communication we are reporting the isolation and functional expression in HeLa cells of two isoforms of the cloned rat brain Na^+–Ca^{2+} exchanger gene. The two isoforms that we have isolated from rat brain cDNA libraries, are both shorter than their homologues the cloned dog [10], human [11] and rat heart [12] Na^+–Ca^{2+} exchanger genes. The open reading frame of one of these cloned 'short' brain isoforms codes for a protein of 935 amino acids and the other of 958 amino acids, as compared to 970, 971 or 973 amino acids coded by the respective cloned dog, rat and human heart Na^+–Ca^{2+} exchanger genes. A 'short' cDNA clone coding for a renal homologue of the cardiac-type exchanger was isolated recently [13], the open reading frame codes for a 940 amino acid long protein. Although the 'finer' mechanistic properties and the regulation of these Na^+–Ca^{2+} exchanger isoforms are yet to be determined, their existence might be the clue for the functional heterogeneities detected in different systems.

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*Accession number of clone RBE-1 is X68812, and of clone RBE-2 X68813.

2. EXPERIMENTAL

Isolation of rat brain homologues of the cardiac Na^+–Ca^{2+} exchanger: a random primed rat brain hippocampus λZAP cDNA library (prepared by J. Boulter from The Salk Institute, La Jolla, CA) and a directionally cloned rat brain cerebellum λZAP cDNA library (prepared by J.-P. Pin at The Salk Institute, La Jolla, CA) were screened several times at low and high stringency [14] with a 2.0 kb fragment (nucleotides 400–2,400) obtained from a rat heart cDNA clone [12]. Each screen of 1.2×10^6 phage plaques resulted in isolation of up to 5 positive clones only. Partial sequencing, of the longest positive clones that were isolated from these libraries indicated, that they were all the rat brain homologues of the cardiac gene, but none were full-length. Further screens using a 0.4 kb fragment (nucleotides 1–400) and a 0.863 kb fragment (nucleotides 1,936–2,799) prepared by PCR (using as templates positive rat brain hippocampus partial clones) did not result in isolation of full-length clones either. Only clones isolated from the hippocampus λZAP cDNA library contained the initiating methionine preceded by an ACAACATG consensus Kozak sequence [15].

Restriction enzyme mapping of the positive clones, sequencing them and comparison to the cloned rat heart exchanger gene indicated, that the first 1,900 nucleotides were identical whereas clones that contained an insert between nucleotides 1,900–2,200 belonged to one of two isoforms.

2.1. *Engineering of full-length clones*

To obtain a full-length gene, we used partially overlapping clones derived from the hippocampus library (positive clones from the cerebellum library did not contain the entire 5′ initiation sequence).

The Na^+–Ca^{2+} exchanger clone RBE-1 was constructed in the fol-

lowing way: clone RBI which contained a 3.7 kbp insert oriented with its coding strand downstream of the T7 RNA polymerase promoter was digested with *Bfr*I and *Sac*I to obtain the pBluescript vector and the initial 1.1 kbp of the exchanger gene. It was ligated to a 1.95 kbp *Bfr*I–*Sac*I fragment obtained from clone 6,922 to construct the full-length clone RBE-1.

The Na^+–Ca^{2+} exchanger clone RBE-2 was constructed by digestion and ligation of two partially overlapping clones RB692 and RBO5. The 3′ 1.8 kb *Xba*I fragment derived from clone RB692 was ligated to an *Xba*I digested RBO5 clone which had 1.37 kb 5′ insert with its coding sequence oriented downstream of the T7 RNA polymerase promoter.

2.2. *Determination of the nucleotide sequence of clones RBE-1 and RBE-2*

Sequencing of the two strands was done by the dideoxy chain termination method [16] using a combination of nested deletions [17] and synthetic oligonucleotide primers designed to close all gaps. Sequenase 2.0 (United States Biochemicals) and the Erase-A-Base kit (Promega Corp., Madison, WI) were used.

2.3. *Expression of Na^+–Ca^{2+} exchange activity*

HeLa cells (ATCC) were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS, 2 mM glutamine and 200 units/ml penicillin/200 μg/ml streptomycin all purchased from Biological Industries (Beth Haemek, Israel). The cells were infected with the recombinant vaccinia virus VTF-7 at a multiplicity of 10 PFU/cell as described [18]. DOTAP (Boehringer-Mannheim, Germany) was used for transfection at a 1:3 plasmid DNA:lipid (w/w) ratio. Cells were grown to near confluency, about 0.5×10^6 cells/well in a 12-well or 0.25×10^6 cells/well in 24-well culture plate or about 5×10^6 cells/80 mm culture dish. 2.5, 1.25 or 10 μg DNA, respectively, was used for transfection. The cells were maintained at 37°C in 5% CO_2 for the times indicated in the specific experiments and assayed for transport activity.

2.4. *Determination of Na^+–Ca^{2+} exchange activity in 'whole' HeLa cells*

At times indicated after transfection, culture media were separated from the cells by aspiration, the cells were overlayed with a solution of 0.14 M NaCl/10 mM Tris-HCl (pH 7.4) for 10 min to preload them with high [Na^+] at room temperature [12]. At the end of the preincubation period, the cells were exposed to 50 μM $^{45}Ca^{2+}$ in either 0.14 M KCl, or 0.14 M NaCl buffered with 10 mM Tris-HCl (pH 7.4) for the times indicated. The reactions were stopped by aspiration of the uptake media and two rapid washes with 0.14 M KCl at 4°C. The cells containing $^{45}Ca^{2+}$ were separated from the culture dishes by the addition of a 0.25% trypsin/0.05% EDTA solution. An aliquot was kept for protein determination [19] and the rest was counted in a liquid scintillation β-counter. Na^+ gradient-dependent Ca^{2+} influx was determined by subtracting the $^{45}Ca^{2+}$ associated with the cells in the absence of a Na^+ gradient (external NaCl) from the amount of $^{45}Ca^{2+}$ associated with the cells in the presence of a Na^+ gradient (external KCl). Each experiment was repeated at least three times. Time points were done in triplicates.

2.5. *Determination of Na^+–Ca^{2+} exchange activity following reconstitution of the expressed proteins in HeLa cells*

At times indicated after transfection, culture media were separated from the cells by aspiration, the cells were washed twice with PBS at 4°C, separated from the culture dish by scrapping, collected by centrifugation at $7,500 \times g$ at 4°C and dissolved in a solution containing brain phospholipids (14 mg/ml) in 0.2 M NaP_i buffer (pH 7.4) and 2% Na^+-cholate. 200 μl of the lipid solution was added to a cell pellet obtained from a 80 mm Petri dish. Cell solubilization and reconstitution was carried out as described [12,20]. To determine the Na^+ gradient component of Ca^{2+} influx, 10 μl of reconstituted HeLa cell proteins were diluted into 350 μl of 0.2 M KCl or NaCl, buffered with 10 mM Tris-HCl (pH 7.4) and containing also 50 μM $^{45}Ca^{2+}$ (1 μCi/ml). The reactions were terminated at times specified by loading the entire reaction mixture onto a Dowex 50 mini-column to separate intravesicular Ca^{2+} from extravesicular one as described [20–22]. Protein content of the reconstituted membrane was determined by the method of Lowry et al. [19]. Net Na^+ gradient dependent Ca^{2+} influx was calculated as for the whole cell assay.

Materials were either Molecular Biology or Analytical grade reagents. Radioactive materials were purchased from Amersham, Life Sciences, UK.

3. RESULTS AND DISCUSSION

The amino acid sequence of clone RBE-2, one of the two isoforms of the rat brain Na^+–Ca^{2+} exchangers is shown in Fig. 1. Clone RBE-1, the second rat brain isoform, is identical to clone RBE-2, except that a segment of 23 amino acids (marked by a bar under the corresponding amino acids of clone RBE-2 in Fig. 1) is missing. Both brain clones, RBE-1 and RBE-2 are also identical to the cloned rat heart Na^+–Ca^{2+} exchanger gene [12], except that they are shorter by 36 and 13 amino acids, respectively, as shown in Fig. 2, where the 'variable' segment of the different cloned Na^+–Ca^{2+} exchanger isoforms is compared. It can be seen, that in both brain clones a stretch of 7 amino acids (636–642) is missing, when compared to the heart or renal exchangers. Then, six highly conserved amino acids (643–648) are present in all the cloned genes, followed by a missing segment of 29 amino acids (649–678) in clone RBE-1 and the renal clone or 6 missing amino acids (649–654) in clone RBE-2. Of the eight 'positive' rat brain clones that contained this 'variable' region of the gene, three were identical to the RBE-1 isoform and five were identical to the RBE-2 isoform. Two of the RBE-1 isoforms were found in the cerebellar library and one in the hippocampus library. All our five RBE-2 isoforms were isolated from the hippocampus library. We did not find in brain cDNA libraries any clones that coded for the 'long' isoform, such as found in rat, dog or human heart cDNA libraries.

Both isoforms of the rat brain Na^+–Ca^{2+} exchanger are functional and electrogenic. Their transport properties were tested after expression in VTF-7 infected HeLa cells. Maximal expression was obtained 18 h after transfection as for the cloned rat heart exchanger [12] (not shown). Na^+ gradient dependent Ca^{2+} influx was determined in RBE-1 or RBE-2 transfected 'whole' HeLa cells, as described in section 2 and [12]; both brain clones had similar Na^+ gradient dependent Ca^{2+} transport activity: 26.4 (S.D. = 4.53; $n = 9$) nmol/mg protein/10 min of uptake for RBE-1, and 31.53 (S.D. = 0.93; $n = 3$) nmol/mg protein/10 min of uptake for RBE-2.

The time course of Ca^{2+} influx into RBE-2 transfected HeLa cells, in the presence and in the absence of a Na^+ gradient, is shown in Fig. 3.

Mechanistic studies we carried out indicated, that the brain synaptic plasma membrane Na^+–Ca^{2+} exchanger is electrogenic [20]. In addition, in brain synaptic mem-

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  1  MLRLSLPPNV SMGFRLVTLV ALLFTHVDHI TADTEAETGG NETTECTGSY
 51  YCKKGVILPI WEPQDPSFGD KIARATVYFV AMVYMFLGVS IIADRFMSSI
101  EVITSQEKEI TIKKPNGETT KTTVRIWNET VSNLTLMALG SSAPEILLSV
151  IEVCGHNFTA GDLGPSTIVG SAAFNMFIII ALCVYVVPDG ETRKIKHLRV
201  FFVTAAWSIF AYTWLYIILS VSSPGVVEVW EGLLTFFFFP ICVVFAWVAD
251  RRLLFYKYVY KRYRAGKQRG MIIEHEGDRP ASKTEIEMDG KVVNSHVDNF
301  LDGALVLEVD ERDQDDEEAR REMARILKEL KQKHPDKEIE QLIELANYQV
351  LSQQQKSRAF YRIQATRLMT GAGNILKRHA ADQARKAVSM HEVNMDVVEN
401  DPVSKVFFEQ GTYQCLENCG TVALTIIRRG GDLTNTVFVD FRTEDGTANA
451  GSDYEFTEGT VIFKPGETQK EIRVGIIDDD IFEEDENFLV HLSNVRVSSE
501  VSEDGILDSN HVSAIACLGS PNTATITIFD DDHAGIFTFE EPVTHVSESI
551  GIMEVKVLRT SGARGNVIIP YKTIEGTARG GGEDFEDTCG ELEFQNDEIV
601  KTISVKVIDD EEYEKNKTFF IEIGEPRLVE MSEKKGGFTL TGQPVFRKVH
651  ARDHPIPSTV ISISEEYDDK QPLTSKEEEE RRIAEMGRPI LGEHTKLEVI
701  IEESYEFKST VDKLIKKTNL ALVVGTNSWR EQFIEAITVS AGEDDDDDEC
751  GEEKLPSCFD YVMHFLTVFW KVLFAFVPPT EYWNGWACFI VSILMIGLLT
801  AFIGDLASHF GCTIGLKDSV TAVVFVALGT SVPDTFASKV AATQDQYADA
851  SIGNVTGSNA VNVFLGIGVA WSIAAIYHAA NGEQFKVSPG TLAFSVTLFT
901  IFAFINVGVL LYRRRPEIGG ELGGPRTAKL LTSSLFVLLW LLYIFFSSLE
951  AYCHIKGF
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Fig. 1. Amino acid sequence of the cloned rat brain Na^+–Ca^{2+} exchanger isoforms. The entire open reading frame of the deduced amino acid sequence of clone RBE-2 is presented. The amino acid sequence of RBE-1 is identical to RBE-2 except that a stretch of 23 amino acids (marked by a bar) is missing.

branes two modes of Na^+–Ca^{2+} exchange, K^+-dependent ('rod outer segment-type') and K^+-independent ('heart-type') co-exist [22]. To find out whether both cloned rat brain isoforms of the Na^+–Ca^{2+} exchanger gene operate in an electrogenic fashion and to answer the question whether any of the brain clones code for a K^+-dependent Na^+–Ca^{2+} exchanger, both cloned exchanger proteins, RBE-1 and RBE-2 were reconstituted (after expression in HeLa cells) into a phospholipid membrane (see section 2). This was done, since preliminary experiments indicated, that valinomycin induced K^+ fluxes could not be demonstrated conclusively in the infected/transfected cells. Reconstitution of the expressed proteins into a synthetic phospholipid membrane ensured that the membrane was tightly sealed and therefore the K^+ flux was amenable to manipulations by valinomycin. Table I shows the apparent initial rate of Ca^{2+} entry into NaP_i preloaded reconstituted vesicles diluted into external KCl with and without valinomycin or into external choline chloride with and without FCCP. Ca^{2+} entry into the vesicles in the absence of a Na^+ gradient (external iso-osmotic NaCl) was also determined and subtracted to determine the Na^+ gradient component of Ca^{2+} flux. It can be seen that addition of valinomycin to a K^+ containing medium increases the apparent initial rate of Na^+ gradient dependent Ca^{2+} influx 4-fold and addition of FCCP to a choline chloride containing medium 2.8-fold indicating that both cloned rat brain Na^+–Ca^{2+} exchanger genes code for proteins that are exchanging Na^+ with Ca^{2+} in an electrogenic fashion. Moreover, since Na^+ gradient dependent Ca^{2+} influx proceeds in the absence altogether of K^+, none of these exchanger isoforms is the K^+-dependent transporter found in synaptic plasma membranes [22].

Control experiments, in which we reconstituted HeLa cell proteins from infected but nontransfected cells, or from noninfected and nontransfected cells, exhibited no endogenous Ca^{2+} fluxes in the presence or absence of a Na^+ gradient, or upon addition of valinomycin to an external KCl-containing medium, or FCCP to an external choline chloride-containing medium.

It is not known at present how the structural differ-

Clone		Length of deletion
Dog heart	E P R L V E M S E K K A L L L N E L G G F T I T - G K Y L Y G Q P V F R K V H A R E H P I P S T V I T I A E E Y D D	1
Human heart	E P R L V E M S E K K A L L L N E L G G F T I T - G K Y L F G Q P V F R K V H A R E H P I L S T V I T I A D E Y D D	1
Rat heart (RHE-1)	E P R L V E M S E K K A L L L N E L G G F T L T E G K K M Y G Q P V F R K V H A R D H P I P S T V I S I S E E Y D D	none
Rat brain (RBE-1)	E P R L V E M S E K K - - - - - - - G G F T L T - E E Y D D	36
Rat brain (RBE-2)	E P R L V E M S E K K - - - - - - - G G F T L T - - - - - - G Q P V F R K V H A R D H P I P S T V I S I S E E Y D D	13
Rabbit kidney	E P K W I R R G M - K A L L L N E L G G F T I T - E E Y D D	30
Amino Acid No.	625	682

Fig. 2. Comparison of the 'variable' segment of the different cloned Na^+–Ca^{2+} exchanger isoforms. A stretch of amino acids (623–682) of the different isoforms of the cloned dog [10], human [11] and rat [12] heart and kidney [13] Na^+–Ca^{2+} exchangers is compared to the cloned rat brain Na^+–Ca^{2+} exchangers. The first initiating methionine is assigned the number **1**, except in the cloned human exchanger [11] where the first methionine is at position **−3** when compared to the other cloned exchangers.

ences between the Na^+–Ca^{2+} exchanger isoforms are translated into functional differences. Hydropathy plot of the amino acid sequence of the cloned rat heart and brain Na^+–Ca^{2+} exchanger genes, using the Kyte and Doolittle [23] program with a window of 20 amino acids (not shown) indicates that the differences between the

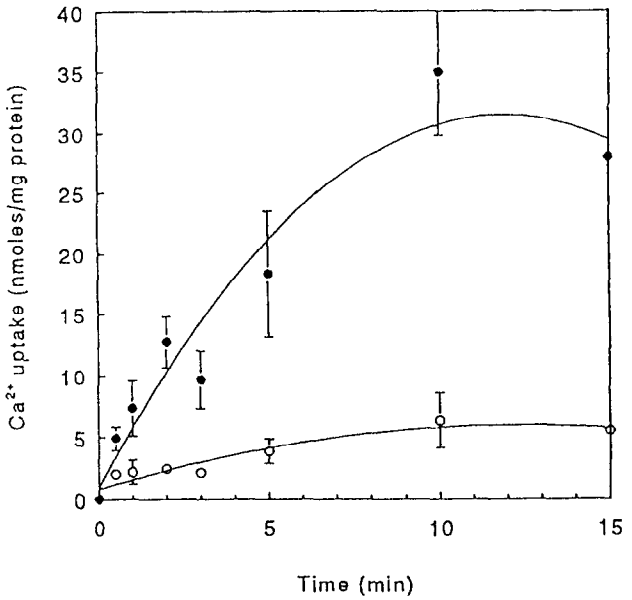

Fig. 3. Time course of Ca^{2+} uptake in transfected HeLa cells. Infected HeLa cells were transfected with clone RHE-2, 1.25 μg DNA/2.5 × 10^5 cells for 16.5 h. At the end of the transfection period, the cells were preloaded with 0.14 M NaCl as described in section 2. $^{45}Ca^{2+}$ uptake was determined in: 0.14 M KCl, 0.01 M Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$ (●); or in 0.14 M NaCl, 0.01 M Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$ (○). Each time point was measured in triplicate; the bars represent standard deviations.

Table I

Electrogenic properties of the cloned rat brain Na^+–Ca^{2+} exchanger isoforms

Clone	External medium	Na^+ gradient dependent Ca^{2+} uptake (nmol/mg protein/min)	S.D.
RBE-1	KCl	8.88	1.246
	KCl + valinomycin	38.45	2.136
	Choline chloride	9.82	0.465
	Choline chloride + FCCP	27.9	0.598
RBE-2	KCl	9.41	0.548
	KCl + valinomycin	38.00	1.866
	Choline chloride	10.35	0.673
	Choline chloride + FCCP	29.83	1.09

HeLa cells were transfected with clone RBE-1 and RBE-2 DNA 10 μg/10^6 cells in a 80 mm culture dish. 17 h after transfection the expressed proteins were reconstituted into a phospholipid membrane (for details see section 2). Ca^{2+} uptake was measured in the 0.2 M NaP_i containing reconstituted vesicles by diluting 10 μl of them into 350 μl of: 0.2 M KCl (without or with 20 μM valinomycin); 0.2 M choline chloride (without or with 20 μM FCCP); or 0.2 M NaCl (with either 20 μM valinomycin or FCCP, and without them). All media contained also 0.1 Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$. Reactions were terminated as described in section 2 and the Na^+ gradient component of the Ca^{2+} flux was calculated by subtracting the amount of Ca^{2+} associated with the vesicles in the absence of a Na^+ gradient from that obtained in its presence.

three isoforms of the cloned rat Na^+–Ca^{2+} exchanger gene are confined to a protein segment of high hydrophilicity. Based on the hydropathy profile and location of potential glycosylation and phosphorylation sites of the cloned canine cardiac exchanger, Nicoll et al. [10,24] proposed that this protein segment is part of the cytoplasmic domain. In an attempt to determine the role of this tentative cytoplasmic domain in regulating Na^+–Ca^{2+} exchange activity, Nicoll and Philipson [24] deleted 439 amino acids (240–679) of the cloned dog heart Na^+–Ca^{2+} exchanger gene. Expression of this mutated exchanger in *Xenopus* oocytes resulted in lower transport activity, suggesting that the cytoplasmic domain has a role in regulation of the antiport. Further experiments, however, in which discrete segments of the cloned gene will be selectively deleted have to be done, to understand the structural basis of Na^+–Ca^{2+} exchange activity and the regulation of its expression in heterologous systems.

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