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Cloning of the rat heart Na^+-Ca^{2+} exchanger* and its functional expression in HeLa cells

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A functional rat heart Na^+-Ca^{2+} exchanger gene has been obtained by splicing and ligating two partially overlapping clones isolated from a rat heart λZAP cDNA library. The deduced primary structure of the protein encoded by the open reading frame corresponds to 971 amino acids, that can be organized into 12 transmembrane helices. The cloned gene was functionally expressed in HeLa cells. Maximal expression was detected 18 h after transfection, after which transport activity rapidly declined. The electrogenic properties of the cloned transporter were demonstrated following reconstitution of the expressed exchanger protein into a tightly sealed phospholipid membrane.

Calcium; Transport; Na^+-Ca^{2+} exchanger; Cloning; Expression; HeLa cells

1. INTRODUCTION

The regulation of intracellular Ca^{2+} ion concentration is of fundamental importance to excitable cell function. The Na^+-Ca^{2+} exchanger is a major Ca^{2+} transporting protein present in all excitable and many non-excitable cells and it contributes to the maintenance of Ca^{2+} homeostasis. Although its existence has been established about 25 years ago, many questions relating to its mechanism of action and regulation are still not fully understood [1,2].

Cloning of transport proteins and expressing them in heterologous systems provides the necessary means for studying the structural basis of transport processes and their cellular regulation. In this report, we describe the cloning of the rat heart Na^+-Ca^{2+} exchanger gene and its functional expression in HeLa cells. The expression system described here provides an easy to handle experimental tool, where the transport properties of the cloned Na^+-Ca^{2+} exchanger can be studied not only in the whole transfected cell but also following its functional reconstitution into an exogenous phospholipid membrane.

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*The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number no. X68191 RNSCEX.

2. MATERIALS AND METHODS

2.1. *Isolation of rat heart Na^+-Ca^{2+} exchanger clones*

A randomly primed λZAP cDNA library obtained from J. Boulter and S.F. Heinemann, The Salk Institute, La Jolla, CA. was screened at high stringency [3] with a 865 bp probe amplified by PCR from rat heart cDNA, using as primers two oligonucleotides based on the published sequence of the dog heart Na^+-Ca^{2+} exchanger and spanning nucleotides 1936 to 2799 of the dog gene [4]. Partial sequencing [5] of positive clones indicated that they are the rat homologues of the canine cardiac exchanger. Since none of these clones was full-length and since two more library screens did not result in isolation of full-length clones either and since sequencing of overlapping segments of the different clones indicated that they were all identical, two partial clones p18 (−63–1150) and p9 (740–3090) were spliced and ligated to obtain the full-length clone RHE-1 (p189).

2.2. *Engineering of a full length rat heart Na^+-Ca^{2+} exchanger*

A 2.1 kb long *Xho*I (Klenow filled)–*Bfr*I fragment, prepared from clone 9, was ligated to *Bfr*I–*Sma*I linearized clone p18. Plasmid preparation, restriction enzyme digestion, purification of digests, ligation, transformation of *E. coli* DH5αF′ and ampicillin selection of transformants were done by standard procedures [6].

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 U/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 [7]. DOTAP (Boehringer Mannheim, Germany) was used for transfection at a 1:3 RHE-1 DNA/lipid (w/w) ratio. Cells were grown at 37°C in 5% CO_2 to near confluency, about 10^6 cells/well in a 6-well culture plate or about 5×10^6 cells/80 mm Petri dish. 5 μg and 10 μg DNA, respectively, were used for transfection.

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 washed twice by PBS at 4° and 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. 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 5 ml 0.14 M KCl at 4°C. The $^{45}Ca^{2+}$-containing cells were separated from the culture dishes by the addition of 0.5 ml of a 0.25% trypsin–0.05% EDTA solution. An aliquot was kept for protein determination [8] 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.

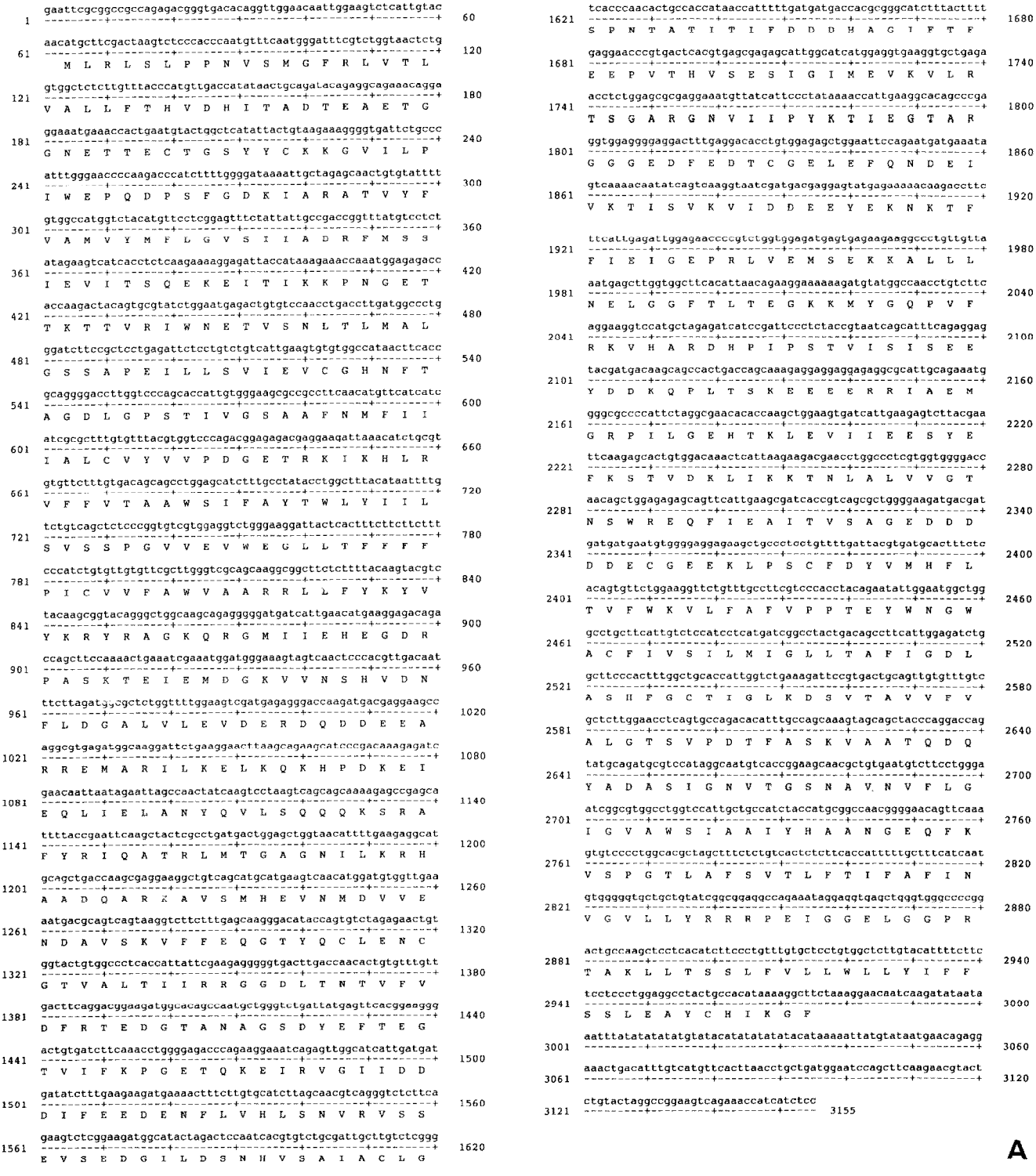


Fig. 1. (A) Nucleotide and amino acid sequence of the cloned rat heart Na^+-Ca^{2+} exchanger.

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 7500 × *g* at 4°C and dissolved in a solution containing brain phospholipids (20 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 [9]. 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) 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 [9,10]. Protein content of the reconstituted membrane was determined by the method of Lowry et al. [11]. Net Na^{2+} gradient-dependent Ca^{2+} influx was calculated as for the whole cell assay.

2.6. *Determination of the nucleotide sequence of clone RHE-1*

Both strands of clone RHE-1 were sequenced using a combination of the dideoxy chain termination method [5] and nested deletions [12] using the Erase-a-Base (Promega Corporation, Madison, WI) kit and synthetic oligonucleotide primers designed to close all gaps. T7 DNA polymerase (Sequenase 2.0 from USB) was used.

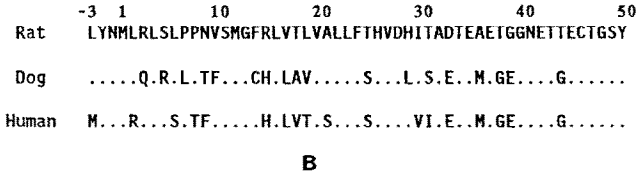
3. RESULTS AND DISCUSSION

The nucleotide sequence and the deduced primary structure of the cloned rat heart Na^+-Ca^{2+} exchanger are shown in the first and second row of Fig. 1A. Starting from the initiating methionine, which is preceeded by a consensus initiation site [13] ACAACATG, the open reading frame of the rat heart Na^+-Ca^{2+} exchanger encodes a protein 971 amino acids long which differs from both the canine [4] and human [14] heart Na^+-Ca^{2+} exchangers by 44 amino acids, 18 of which are found in the 5′ region of the gene, in the putative [15] signal peptide and NH_2 terminus of the protein (Fig. 1B). It should be noted that the overall lengths of the canine heart exchanger is 970 amino acids (starting from the initiating methionine) the human one is 973 amino acids and the rat heart exchanger is 971 amino acids. But whereas the human gene has three extra amino acids within the putative signal peptide sequence [15], the rat heart exchanger contains an extra amino acid at position 649 (Fig. 1C).

Hydropathy profile of the cloned rat heart exchanger, using the Kyte and Doolittle program [16] over a window of 20 amino acids indicates (see Fig. 1D), that the protein can be organized into 12 transmembrane helices.

Expression of Na^+-Ca^{2+} exchange activity by the cloned rat heart gene was tested in HeLa cells. Since the coding sequence of the gene was cloned down-stream of the T7 RNA polymerase promoter in the pBluescript (Stratagene) vector, infection of the cells with the recombinant vaccinia virus VTF-7 prior to transfection, was used to introduce the T7 RNA polymerase gene into HeLa cells [7]. The transport assay system involved creation of an outward oriented (high inside) Na^+ gradient which was used to drive Ca^{2+} influx. This was done in two ways: (1) by preincubation of whole HeLa cells with high [Na^+] containing solutions (see section 2); and (2) by introduction of high [Na^+] into proteoliposomes in the course of reconstitution of the HeLa cell proteins.

Preliminary experiments indicated, that preincubation of transfected HeLa cells with an isoosmotic high Na^+ containing solution for 10 min was sufficient to



B

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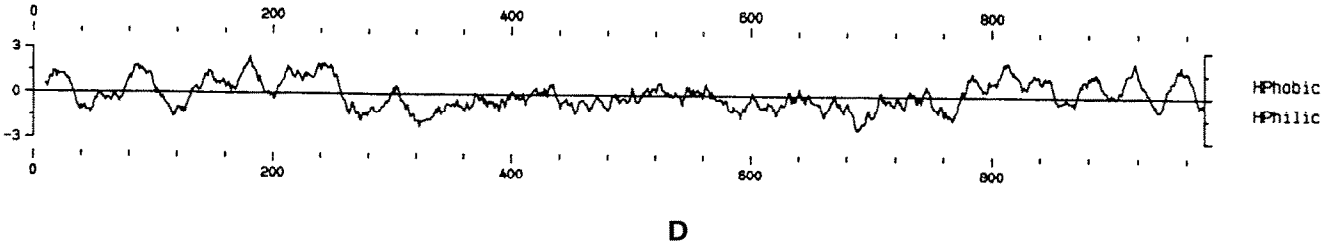

D

Fig. 1. (B) The NH_2-termini of the rat, dog and human Na^+-Ca^{2+} exchangers: The initiating methionine in the rat and canine genes is no. 1. To preserve the same numbering, the first methionine in the human gene [14] is labeled −3. Only different amino acids in the canine and human exchanger are marked. Each dot indicates an identical amino acid. (C) Comparison of the deduced amino acid sequences in segment 638–657 of the rat, dog and human Na^+-Ca^{2+} exchangers. (D) Hydropathy profile of the cloned rat heart Na^+-Ca^{2+} exchanger.

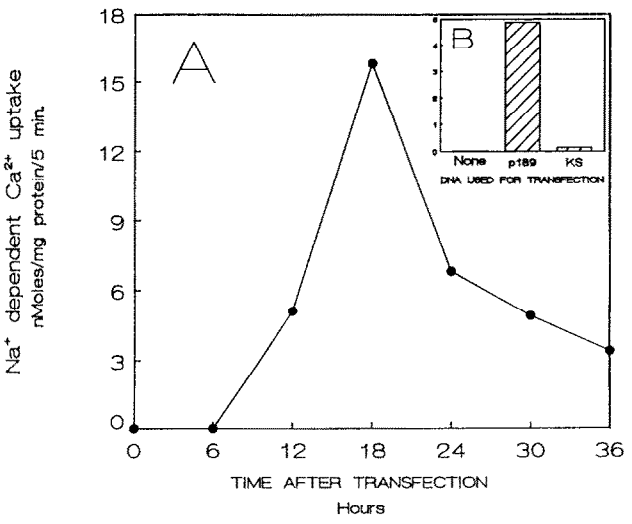

Fig. 2. (A) Time course of expression of Na^+ gradient dependent Ca^{2+} influx in HeLa cells. Infected HeLa cells were DNA-transfected as described in section 2. At times specified, Na^+ gradient dependent Ca^{2+} influx was determined. (B) 15 h after transfection Na^+ gradient-dependent Ca^{2+} uptake was measured for 5 min as in Fig. 2A in control-infected only HeLa cells, and in infected HeLa cells transfected with RHE-1 DNA or with pBluescript KS DNA.

increase intracellular [Na^+]. Neither the addition of nystatin nor the inclusion of ouabain in the high Na^+ containing preincubation solution led to significantly higher Ca^{2+} influx than that obtained following preincubation of the transfected cells for 10 min alone (not shown). However, omission of the preincubation step with the high [Na^+]-containing solution prevented Ca^{2+} influx into transfected HeLa cells, indicating that Ca^{2+} influx depended on the high inside Na^+ concentration.

Fig. 2A shows the time course of expression of Na^+ gradient driven Ca^{2+} influx in transfected HeLa cells. Na^+ loaded HeLa cells were exposed to $^{45}Ca^{2+}$ in the presence (external isoosmotic KCl solution) or in the absence (external NaCl solution) of an inward/outward oriented Na^+ gradient. The Na^+ gradient-driven Ca^{2+} influx at different times after transfection is shown. The uptake reaction was carried out for 5 min. It can be seen that up to 6 h after transfection no transport activity can be detected. Between 6 and 12 h, expression of Na^+ gradient driven Ca^{2+} influx starts and it increases four-fold between 12 and 18 h after transfection. Between 18 and 24 h after transfection a rapid decrease in transport activity is obtained which is followed by a slower phase of decrease. 36 h after transfection only 20% of the maximal transport activity is detected. No endogeneous Na^+ gradient dependent Ca^{2+} transport activity was detected in infected-nontransfected HeLa cells, or in HeLa cells transfected with pBluescript KS DNA only (Fig. 2B).

The time course of the $^{45}Ca^{2+}$ uptake reaction itself is shown in Fig. 3. In this experiment, the transfection was carried out for 15 h, after which the cells were tested for expression of transport activity. It should be noted, that after 5 min, no further Ca^{2+} is taken up by the transfected cells. It is quite possible, that this is a result of the dissipation of the driving Na^+ gradient.

Reconstitution of HeLa cell proteins into phospholipid membranes for transport assays offers several advantages over whole cells. First, the transport activity measured in intact cells depends on the maintenance of ionic gradients across the cell membrane. Since infected/transfected HeLa cells can be easily preloaded with high [Na^+] in the absence of any added Na^+ ionophore (e.g. nystatin) and without inhibition of the Na^+-K^+ ATPase it seems, that their plasma membrane is permeable to Na^+ and maybe other monovalent ions. Hence, it was not surprising, that addition of valinomycin to the K^+ containing medium or FCCP to a choline chloride containing external medium had only a minimal effect (less than 10% on the initial rate) on the rate of transport when transfected whole HeLa cells were used for the assay. However, using the reconstituted system, we were able to demonstrate the electrogeneity of the antiport which is shown in Fig. 4. It can be seen, that the basal initial rate (closed circles) of Ca^{2+} influx in external K^+ containing solution increases about 5-fold when valinomycin is added to the medium (closed squares). Valinomycin had no effect in external isoosmotic NaCl solution when no net Ca^{2+} influx was obtained (open circles and open diamonds). The importance of the driving Na^+ gradient is demonstrated also when Nigericin, which exchanges internal Na^+ with the external K^+ is added to the KCl containing medium even in the presence of valinomycin (open squares). Similar stimulation of the rate of Na^+ gradient dependent Ca^{2+} influx was obtained when FCCP was added to an external choline

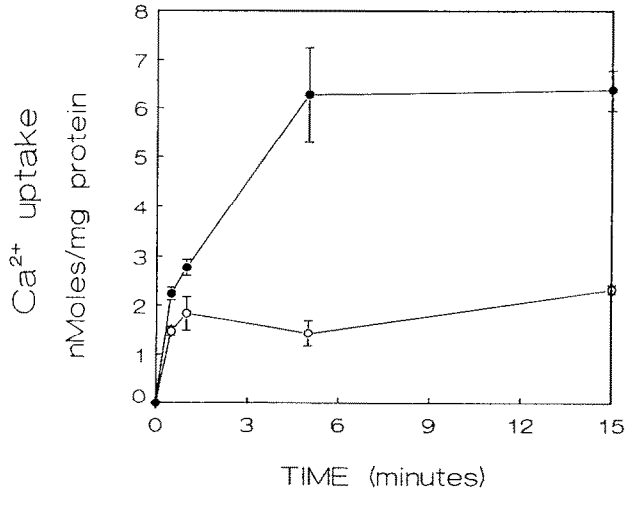

Fig. 3. Time course of Na^+ gradient dependent Ca^{2+} uptake in transfected HeLa cells. Infected HeLa cells were transfected with 5 $\mu g/10^6$ cells of RHE-1 DNA for 15 h. At the end of the transfection period, $^{45}Ca^{2+}$ uptake in the presence of a Na^+ gradient and in its absence was measured in triplicates for the times specified. (●), external KCl solution; (○), external NaCl solution.

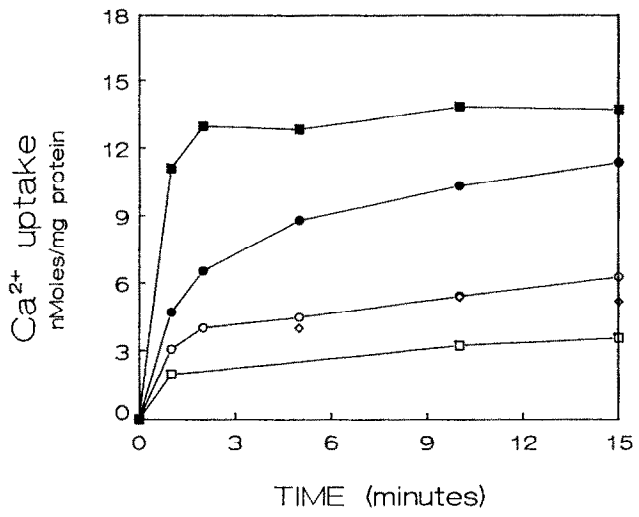

Fig. 4. Stimulation of Na^+ gradient dependent Ca^{2+} uptake in RHE-1 transfected HeLa cells by valinomycin. Infected HeLa cells were transfected for 19 h with RHE-1 DNA. Following reconstitution (see section 2), 10 μl of reconstituted membranes were diluted into 350 μl of either: 0.2 M KCl, 0.01 M Tris-HCl pH 7.4, and 50 μM $^{45}CaCl_2$ (●) or the same medium except that it contained also 20 μM valinomycin (■), or 20 μM nigericin (□); 0.2 M NaCl, 0.01 M Tris-HCl pH 7.4, 50 μM $^{45}CaCl_2$ with (◇) and without 20 μM valinomycin (○) is also shown.

chloride containing medium (not shown). In addition, since we know [17] that the intravesicular volume of the reconstituted vesicles is 1.14 μl/mg phospholipid, and that the protein to phospholipid ratio is 1:25, we can calculate the intravesicular $[Ca^{2+}]$. In these experiments it was 0.45 mM, a 9-fold concentration over the external 50 μM.

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