The Ca^{2+} Antagonists Binding Cytosolic Protein Has Properties of the Ca^{2+} Channel

O. Križanová, O. Hurňák and S. Hudecová

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Czechoslovakia

Abstract. In our previous work (Križanová et al. 1989) we have described a protein from rabbit skeletal muscle cytosolic fraction, which is able to bind dihydropyridines and phenothiazines. In the present work conclusive evidence is provided for the ability of the phospholipid-reconstituted cytosolic protein to transport calcium. The calcium transport was stimulated by BAY K 8644 and inhibited in the presence of PN 200-110. Our observations were confirmed also by electrophysiological measurements on planar lipid bilayers. The possibility that the cytosolic fraction was contaminated with membranes could be definitely ruled out. Nevertheless, the nature of the protein under study is still in the frame of guess.

Key words: Ca^{2+} antagonist binding — Calcium channel — Calcium transport — Cytosolic protein — Planar lipid bilayer

Abbreviations:
DHP — dihydropyridine; CHAPS — 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate; Pi — inorganic phosphate; EDTA — ethylenediamine tetraacetic acid; Hepes — 4-(2-hydroxyethyl) piperazine — 2-ethanesulfonic acid; PMSF — phenylmethylsulphonyl fluoride

Introduction

In our previous work, we have described a protein isolated from rabbit skeletal cytosolic fraction with molecular weight of approx. 90,000 which bound Ca^{2+} antagonists (Križanová et al.1989). Also, some other experiments have indicated a relationship between the protein and the DHP receptor, and/or its alpha 1 subunit. The proposal that this protein could be the cytosolic precursor of calcium channel is not in agreement with the fact that the cDNA encoding the alpha 1 subunit of the dihydropyridine receptor has 6083 nucleotides, and the whole protein is expressed in 1873 aminoacids (Tanabe et al. 1987; Ellis et al. 1988; Morton and Froehner 1989). The low molecular weight obtained (90K)

allows to assume that the cytosolic protein may be a metabolic product of the DHP receptor alpha subunit. Therefore, we studied some more properties of this protein, and compared them with the properties of the dihydropyridine receptor.

Materials and Methods

Materials

^{3}H PN 200-110 (specific activity 3.15 TBq/mmol) and ^{3}H azidopine (specific activity 1.7 TBq/mmol) were from Amersham (UK). $^{45}CaCl_2$ (specific ativity 129 GBq/g Ca^{2+}) was from Institute of Atomic Energy, Poland. Low molecular weight calibration kit was from Pharmacia, azolectin was obtained from Merck. Bray's scintillation coctail was from Spolana Neratovice, Czechoslovakia. All other chemicals were from Serva (FRG) or Lachema (Czechoslovakia), and were of analytical grade.

Methods

Cytosolic fraction was prepared from rabbit skeletal muscle after precipitation of proteins with ammonium sulphate as described by Križanová et al. (1989). All isolation steps were performed in isolation buffer (20 mmol/l Tris-HCl pH 7.0, 0.3 mol/l sucrose, 1 mmol/l iodacetamide, 1 mmol/l PMSF, 1 mmol/l phenantroline and 1 mmol/l benzamidine). After removing the membranes by centrifugation at 150,000 × *g*, ammonium sulphate was added to the supernatant to 50% saturation. The solution was mixed for 1 hour at 4 °C. The desalted proteins were sedimented by centrifugation (50,000 × *g* for 30 min.) and the sediment was dissolved in a small volume of 50 mmol/l Hepes-NaOH, pH 7.4 and 1 mmol/l $CaCl_2$. Na^+/K^+ ATPase activity was determined according to Norris and Cary (1981) as the difference between total and ouabain sensitive ATPase. Protein was determined by the modified method of Lowry et al. (1951).

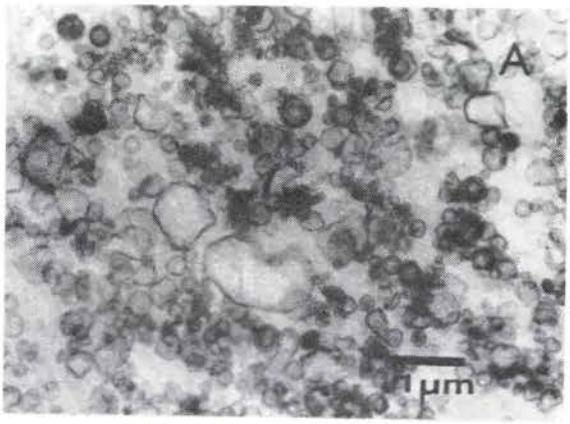
Reconstitution of proteins from the cytosolic fraction

Azolectin was dissolved in buffer Y (50 mmol/l Hepes-NaOH, pH 7.4, 1 mmol/l $CaCl_2$, 2% CHAPS) and then mixed with the same volume of the protein solution (in an azolectin to proteins ratio of 50:1). The mixture was passed through a Sephadex G-50 column to remove CHAPS. The column was washed with 50 mmol/l Hepes-NaOH, pH 7.4, 1 mmol/l $CaCl_2$ and opalescent fractions were collected. The obtained proteoliposomes were sonicated 3 × 15 seconds and used for transport studies.

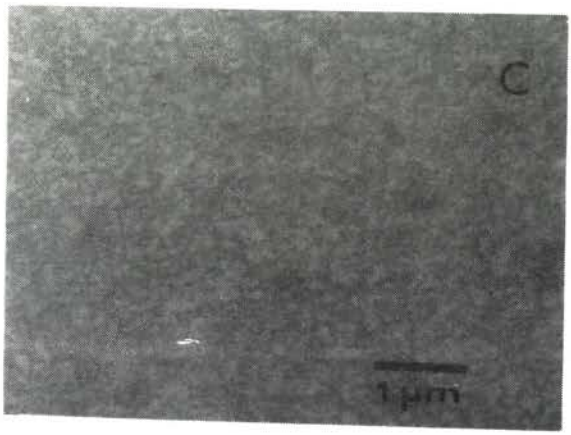
$^{45}Ca^{2+}$ transport into proteoliposomes

$^{45}Ca^{2+}$ transport was measured in buffer T (50 mmol/l Hepes-NaOH, pH 7.4, 25 mmol/l $CaCl_2$, 125 mmol/l Na^+ or K^+, final volume 200 μl). The concentration of radioactive calcium was 1 μmol/l.

Fig. 1. Electron microscopy of membrane fraction (*A*) (magnification 6000 ×), supernatant centrifuged at 500,000 × *g* (*B*) (magnification 7500 ×), and of supernatant precipitated with ammonium sulphate (*C*) (magnification 7500 ×). Typical membrane vesicles can be seen in *A*; few reticular membranes are present in *B*, and there is only a homogenous mass without any membrane contamination in *C*.


A
1 μm


B
1 μm


C
1 μm

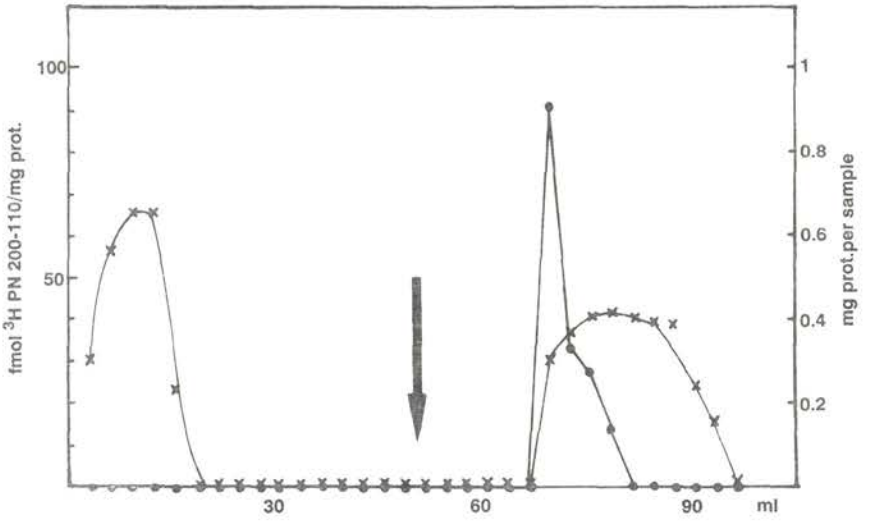


Fig. 2. Protein (x—x) and radioactivity (• •) elution profiles from purified (on DEAE-Sepharose) cytosolic fraction. The radioactivity appears after elution of proteins with 0.5 mol/l sodium chloride.

The transport of calcium was determined as the difference between the values obtained under depolarizing (125 mmol/l K^+ and nondepolarizing (125 mmol/l Na^+) conditions. BAY K 8644 (5 μmol/l, 10 μl) or PN 200-110 (100 μmol/l, 10 μl) was added as the stimulation and inhibition agent respectively. After 1 min incubation, the fractions were passed through a Sephadex CM column, washed with 2 ml of buffer T. Radioactivity was measured on a Rackbeta counter after addition of Bray's scintillation coctail.

Planar lipid bilayer measurements

Planar lipid bilayer measurements were performed as described by Rosenberg et al. (1988) with some modifications. Planar lipid bilayers were formed from *n*-decane (Fluka) solution of azolectin (Fluka) (40 mg/ml), across a circular aperture (600 μm in diameter) in a Teflon wall separating two chambers containing identical solutions (50 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l Hepes NaOH, pH 7.4). Membrane thinning was monitored optically. After bilayer formation $BaCl_2$ was added (from 1 mol/l stock) to the *cis* chamber to a final concentration of 100 mmol/l, and 10 μmol/l BAY K 8644 (from 5 mmol/l stock in ethanol) was added to both chambers. T-tubule vesicles were added to the *cis* chamber. After stirring, the bilayer was broken and reformed.

With the use of an agar bridge electrode, the *cis* chamber was connected to a voltage command signal, the *trans* chamber was connected to a current-voltage converter circuit (15 GΩ feedback resistor). Currents were filtered at 250 Hz, digitized at 500 Hz and stored in a SM 4-20 computer for later analysis. All measurements were performed at steady state holding potential. The recordings were analyzed with a software package developed in our laboratory.

The overlay technique for the determination of the calcium binding proteins

The soluble fraction was separated in 8% SDS gels under reducing conditions (Laemmli 1970). The

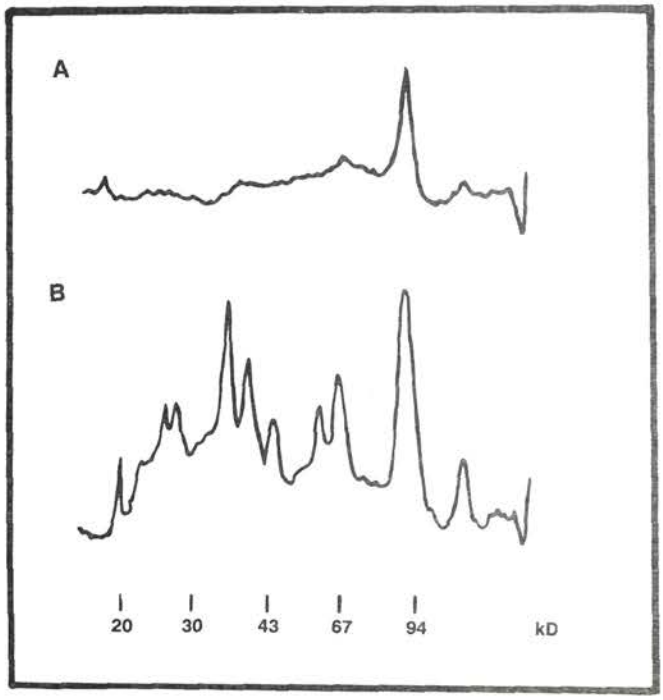


Fig. 3. Densitogram of the protein profile from the cytosolic fraction before (*B*) and after (*A*) purification on DEAE-Sepharose. Purification shows a protein peak in the area of molecular weight 90.000.

gels were fixed in 50% methanol, and Ca^{2+} overlay was done as described by Hincke (1988). After washing in 5% ethanol, the gels were cut into 0.5 cm stripes, and the radioactivity of each stripe was measured.

Results and Discussion

Although the DHP receptors have been well studied (for review see Glossmann and Striessnig 1988; Hofmann et al. 1988) the issues concerning their synthesis and metabolic turnover have not been discussed as yet. This prompted us to try to bind Ca^{2+} antagonists to the cytosolic fraction to reveal some of the metabolic pathways. We could identify the binding sites for these ligands (Križanová et al. 1989). The next step was to exclude membrane contamination. Electron microscopy and the determination of Na^+/K^+ ATPase activity, which is a good marker of the presence of membranes, were employed. The electron microscopi-

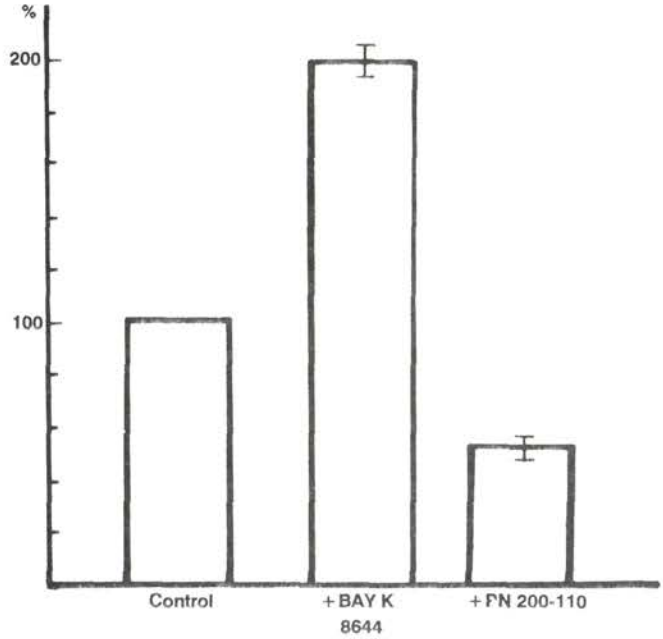


Fig. 4. The cytosolic protein fraction reconstituted into asolectin vesicles: measurement of the $^{45}Ca^{2+}$ influx. This influx was taken for 100%. In the presence of BAY K 8644 the influx increased to 200%. Addition of PN 200-110 inhibited $^{45}Ca^{2+}$ influx to 50%.

cal pictures are shown in Fig. 1. The membrane fraction (*A*) formed typical phospholipid vesicles. The Na^+/K^+ ATPase activity was 1.5 μmol Pi/hour/mg protein. When membranes were removed by centrifugation at 150,000 × *g* and the supernatant was further centrifuged at 500,000 × *g*, some residues of ribosomal membranes were seen (*B*). After precipitation of the supernatant with ammonium sulphate a homogenous mass without any vesicles was obtained. No Na^+/K^+ ATPase activity was observed either in fraction B or C. These results clearly show that there was no membrane contamination in the cytosolic fraction obtained by ammonium sulphate precipitation.

For further purification a DEAE-Sepharose column was used. Elution of proteins with 0.5 mol/l sodium chloride yielded a protein fraction which bound PN 200-110 (Fig. 2). Electrophoresis in 8% SDS gel under reducing conditions gave a protein with a molecular weight of approx. 90K (Fig. 3*A*). This molecular weight corresponds to our previous findings. For comparison, Fig. 3*B* shows the densitogram of the electrophoresis of the unpurified fraction.

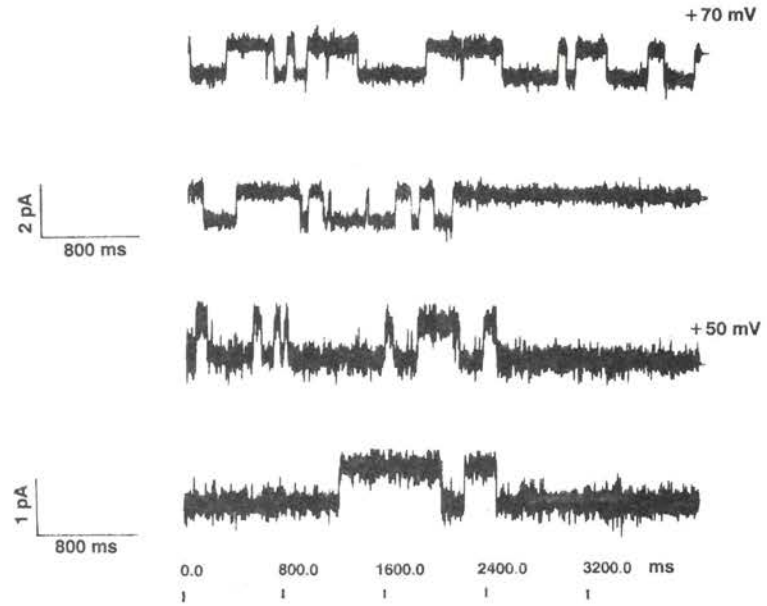


Fig. 5. Single-channel recordings after incorporation into planar lipid bilayers at voltages +50 mV and +70 mV in 50 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l Hepes-NaOH 10μmol/l BAY K 8644 pH 7.4 on both sides, and 100 mmol/l $BaCl_2$ on *cis side*.

One of the properties of the DHP receptor is its ability to transport calcium. The next step therefore included the measurement of the calcium transport by the cytosolic protein after its reconstitution into phospholipid vesicles. The control transport was taken for 100%. BAY K 8644 stimulated the Ca^{2+} transport to 200% and PN 200-110 reduced it to 50% of the control values (Fig. 4). The ability of dihydropyridines to affect calcium transport is an observation in support of the relationship of the protein studied and the DHP receptor. Electrophysiological measurements (Fig. 5) also support this idea, since upon the addition of BAY K 8644 calcium channels from the cytosolic fraction were observed to open at two potentials, +70 mV and +50 mV. The current values of approximately 1 pA are similar to that measured from L-type calcium channel in skeletal muscle (Pelzer et al. 1988; Rosenberg et al. 1988). As a rule, the channel inactivated within 1 min of incorporation into a planar lipid bilayer.

The alpha 1 subunit of the DHP receptor has two calcium binding domains. Very probably, calcium binding sites are also present in the cytosolic protein.

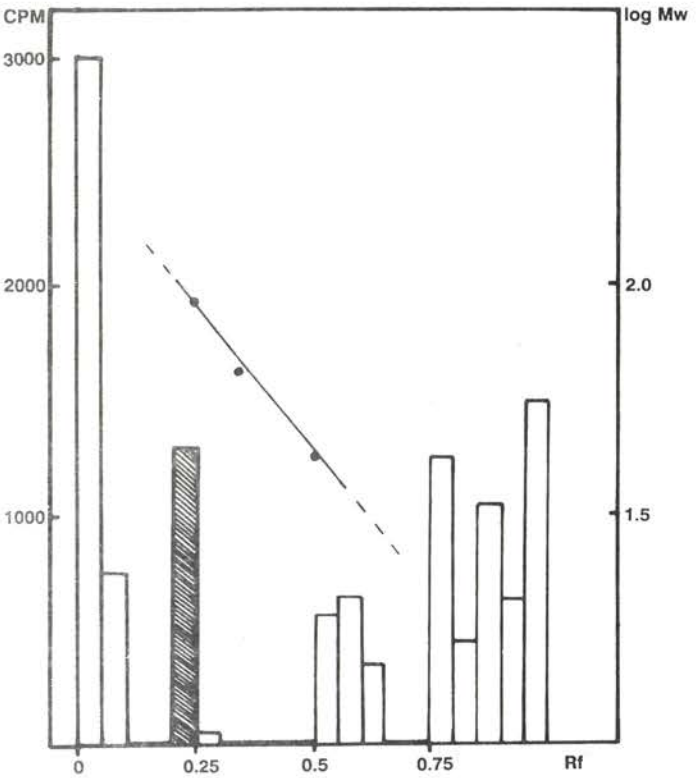


Fig. 6. The result of electrophoresis of the cytosolic fraction with subsequent overlaying of the gels with $^{45}Ca^{2+}$. The black column shows that the protein with the molecular weight of approx. 90,000 is also a calcium binding protein.

This was confirmed by incubating the gels containing the cytosolic protein in the presence of $^{45}Ca^{2+}$. Fig. 6 shows the results of this experiment. The polyacrylamide gel containing the cytosolic fraction precipitated by ammonium sulphate was overlayed with $^{45}Ca^{2+}$, and processed as described above (see Materials and Methods). Radioactive calcium was bound also to the area, where 90K protein appeared. It may be assumed that this protein binds calcium.

The present experiments all suggested a relationship of the 90K cytosolic protein and the alpha 1 subunit of DHP receptor in skeletal muscle. Results published by Tanabe et al. (1987) and Ellis et al. (1988) make it unlikely that this protein is the precursor of the L-type calcium channel.

A comparison of the present data with the experiments done by Johnson et

al. (1988) and Kanngiesser et al. (1988) may suggest that the protein studied is a metabolic product of the DHP receptor. Both groups cleaved the large 160 K alpha subunit with V-8 protease and obtained residues with Mw 85K, 33K and 28K (Johnson et al. 1988) or 81 and 32K (Kanngiesser et al. 1988). The molecular weight of the major residue is similar to that obtained for the calcium binding protein in our experiments. These results allow the assumption concerning metabolic degradation of the DHP receptor alpha subunit.

The cleavage of the protein may occur either in the cytoplasm or in the membrane. This assumption remains to be confirmed. The other possibility, namely that the protein with the properties of the calcium channel, but with unknown function, is not a metabolic product, remains open. Also the question concerning the functional importance of the protein is highly appealing. The cytosolic protein can serve as the carrier for calcium, or it can be involved in some other metabolic regulation process. All the above hypotheses require experimental evidence.

Acknowledgement. The authors wish to thank Prof. J. Zachar for suggesting the topic, stimulating discussions and critical comments on the manuscript; Dr. Marta Novotová for performing electron microscopical examinations; and Mrs. D. Chalupková for technical assistance.

References

Ellis S. B., Williams M. E., Ways N. R., Brenner R., Sharp A. H., Leung A. T., Champbell K. P., McKenna E., Koch W. J., Hui A., Schwartz A., Harpold M. M. (1988): Sequence and expression of mRNAs encoding the alpha 1 and alpha 2 subunits of a DHP-sensitive calcium channel. Science **241**, 1661–1664

Glossmann H., Striessnig J. (1988): Structure and pharmacology of voltage-dependent calcium channels. ISI Atlas Sci. Pharmacol. 202–210

Hienke M. T. (1988): Conditions for improved adsorption of calmodulin to nitrocellulose: Detection by $^{45}Ca^{2+}$ binding. Electrophoresis **9**, 303–306

Hofmann F., Oeken H. J., Schneider T., Sieber M. (1988): The biochemical properties of L-type calcium channels. J. Cardiovasc. Pharmacol. **12**, S25–S30

Johnson D. F., Kuo T. H., Giacomelli F., Wiener J. (1988): Structural analysis of the calcium channel by photoaffinity labelling and limited proteolysis. Biochem. Biophys. Res. Commun. **154**, 455–461

Kanngieser U., Nalik P., Pongs O. (1988): Purification and affinity labelling of dihydropyridine receptor from rabbit skeletal muscle membranes. Proc. Nat. Acad. Sci. USA **85**, 2969–2973

Križanová O., Hudecová S., Orlický J., Zachar J. (1989): Is Ca^{2+} antagonists binding protein from cytosolic fraction the precursor of alpha 1 subunit of Ca^{2+} channel? Gen. Physiol. Biophys. **8**, 99–111

Laemmli U. K. (1970): Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685

Lowry O. H., Rosebrough N. J., Farr A. L., Randal R. J. (1951): Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**, 256–272

Morton M. E., Froehner S. C. (1989): The alpha 1 and alpha 2 polypeptides of the dihydropyridine sensitive channel differ in developmental expression and tissue distribution. Neuron **2,** 1499—1506

Norris D. M., Cary L. (1981): Properties and subcellular distribution of Na^+/K^+-ATPase and Mg^{2+}-ATPase in the antennae of *Periplaneta Americana*. Insect Biochem. **11,** 743—750

Pelzer D., Cavalie A., Flockerzi V., Hofmann F., Trautwein W. (1988): Reconstitution of Solubilized and Purified Dihydropyridine Receptor from Skeletal Muscle Microsomes as Two Single Calcium Channel Conductances with Different Functional Properties. In: The Calcium Channel: Structure, Function and Implications. (Eds. M. Morad, W. Nayler, S. Kazda, M. Schramm). Springer Verlag, Berlin—Heidelberg

Rosenberg R. L., Hess P., Tsien R. W. (1988): Cardiac calcium channels in planar lipid bilayers. L-type channels and calcium permeable channels open at negative membrane potentials. J. Gen. Physiol. **92,** 27—54

Tanabe T., Takeshima H., Mikami A., Flockerzi V., Takahashi H., Kangawa K., Kojima M., Matsuo H., Hirose T., Numa S. (1987): Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature **328,** 313—318

Final version accepted February 14, 1990