Volume 250, number 2, 509–514 FEB 07320 July 1989

Evidence for the existence of a cardiac specific isoform of the α_1 subunit of the voltage dependent calcium channel

Donald F. Slish, Dorothy B. Engle, Gyula Varadi, Ilana Lotan*, Datna Singer*, Nathan Dascal* and Arnold Schwartz

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0575, USA and **Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel*

Received 10 May 1989

Biochemical, pharmacological and electrophysiological evidence implies the existence of tissue specific isoforms of the L-type VDCC. The α_1 and α_2 subunits of the skeletal muscle calcium channel have been previously cloned and their amino acid sequence deduced. Here we report the isolation and sequencing of a partial cDNA that encodes a heart specific isoform of the α_1 subunit. The amino acid sequence deduced from this part cDNA clone shows 64.7% similarity with the skeletal muscle α_1 subunit. Northern analysis reveals 2 hybridizing bands, 8.5 and 13 kb, in contrast to one 6.5 kb band in the skeletal muscle. Selective inhibition of mRNA expression in *Xenopus* oocytes by complementary oligodeoxynucleotides derived from the heart clone provides further evidence that the cDNA corresponds to an essential component of the VDCC. These data further support the existence of tissue-specific isoforms of the L-type VDCC.

Ca^{2+} channel, voltage-dependent; Dihydropyridine receptor; Ion channel; cDNA cloning

1. INTRODUCTION

Based upon electrophysiological and pharmacological data, at least 3 distinct isoforms of a VDCC have been postulated [1–3] and designated N-, T- and L-types [4]. The L-type VDCC is characterized by its sensitivity to a pharmacologically important group of drugs called calcium antagonists [5,6]. These drugs are widely used in the treatment of cardiovascular diseases such as coronary artery disease, supraventricular arrhythmia, and hypertension [7,8]. The skeletal muscle L-type VDCC has been extensively studied and its putative structure consists of 5 polypeptides, designated α_1, α_2, β, γ, and δ [9,10]. The α_1 subunit contains the calcium antagonist drug binding domains [9,11]; this subunit has frequently been called the 'DHP receptor'. Electrophysiological and pharmacological data are consistent with the presence of tissue specific isoforms of the L-type VDCC, in skeletal muscle, brain, heart, and vascular smooth muscle. The α_1 subunit from skeletal muscle has been cloned and sequenced [12,13]. Using molecular cloning and Northern analysis, we present further evidence in favor of the existence of a heart specific isoform of the α_1 subunit of the VDCC.

Correspondence address: A. Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0575, USA

Abbreviations: VDCC, voltage-dependent calcium channel; DHP, 1,4-dihydropyridine

2. MATERIALS AND METHODS

A λgt10 library, made from cDNA size selected from 4.5 to 8.0 kb, was obtained from Dr David McLennan (University of Toronto); 10^6 plaques were screened with a 4.3 kb *Eco*RI/*Sac*I fragment of the skeletal muscle α_1 clone [13] by standard screening techniques [14]. Filters were prehybridized in a solution of 50% formamide, 5 × STE (1 × STE: 100 mM NaCl,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

10 mM Tris-HCl, pH 8.0), 0.1% SDS, 5 × Denhardt's solution, and 200 mg/ml denatured salmon sperm DNA. Hybridization was carried out in the same solution with the addition of 10^6 dpm/ml radioactively labeled probe [15]. Final filter washes were done in 1 × SSC (150 mM NaCl, 10 mM sodium citrate, pH 7.0), 0.1% SDS and 0.1% sodium pyrophosphate.

Northern analysis was performed by methods previously described [14]. Total RNA was prepared by the procedure of Chomczynski and Sacci [16] and poly(A^+) RNA was isolated by oligo(dT) affinity chromatography [17]. Total or poly(A^+) RNA was electrophoresed in denaturing formaldehyde agarose gels and transferred to Nytran membranes (Schleicher and Schuell) according to the manufacturer's protocol. Washing conditions are stated in the figure legends.

Sequencing of the cDNA clones was carried out by the dideoxynucleotide chain termination method [18]. Sequenase (US Biochemical) and T7 polymerase (Pharmacia) reagent kits were used in addition to the method described. Sequence comparisons were performed by DNanalyze program [19].

Deoxyoligonucleotide probes were synthesized by our DNA Core Facility with an automated DNA synthesizer (Applied Biosystems). The techniques of preparation and injection of oocytes, selective inhibition of RNA expression in oocytes (hybrid arrest of translation), and measurement of calcium currents have been previously described [20].

3. RESULTS

In order to isolate a cardiac specific isoform of the DHP receptor, a cardiac λgt10 cDNA library was screened with a skeletal muscle DHP receptor probe (4.3 kb *Eco*RI/*Sac*I fragment) which contained most of the coding region. One clone, 3.4 kb in length (fig.1, HTDHP 1.0), was originally isolated and sequenced. A fragment of this clone closest to the 5′ end (a 1.35 kb *Bgl*II fragment) was then radioactively labeled and used to rescreen the library. Several additional clones were isolated, the longest of which was 4.6 kb in length (fig.1, HTDHP 2.0). Restriction mapping and partial sequencing showed that the overlapping regions of this clone were identical to HTDHP 1.0, and it covered an additional 1.2 kb in the 5′ direction.

In order to confirm the functional identity of the cDNA clone, we used the approach of hybrid arrest of mRNA expression in *Xenopus* oocytes by complementary oligodeoxynucleotides [20]. To this end, 3 complementary oligonucleotides were synthesized; one of these (DHPRH-O_1) is complementary to the putative S4 voltage sensor region of Motif 4 (fig.2). Two others (DHPRH-O_2 and -O_3) were derived from the C-terminal region of the partial heart cDNA clone. These oligonucleotide probes were hybridized to total cellular RNA isolated from rabbit heart. Then, aliquots of the hybridization mixture were injected into oocytes and 48 h later the calcium currents were measured. As the data summarized in table 1 indicate, all 3 probes significantly inhibited the Bay K 8644-sensitive currents elicited by the injection of heart RNA into oocytes. On the basis of Bay K 8644 sensitivity, this current is identified as being carried by the L-type VDCC [4]. DHPRH-O_2 did not inhibit sodium current produced by injection of RNA from mouse brain (1507 ± 282 nA, control, 1327 ± 299 nA, DHPRH-O_2 25μg/ml).

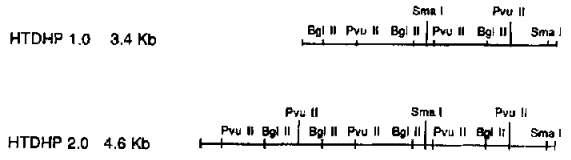

Fig.1. Restriction maps of isolated rabbit heart clones. The clones are represented from the 5′ end to the 3′ end.

The deduced amino acid sequence of HTDHP 2.0 shows 64.7% similarity with the skeletal muscle DHP receptor (fig.2). Much of the similarity lies in the area of the membrane spanning regions. The heart isoform shows 68.7% similarity in the region of the skeletal muscle α_1 comprised of residues 617–1385, while the remaining C-terminal sequence shows only 56.3% similarity. It is also notable that the last 135 amino acid residues of the skeletal muscle α_1 are completely divergent from the heart isoform. Furthermore, the heart clone encodes a C-terminus of 73 additional amino acids.

Within the membrane-associated domains, it is of interest that the similarity is greatest in stretches proposed to be embedded in the membrane, while intracellular and extracellular loops are more variable. One especially variable region is the extracellular loop between the S3 and S4 segments of Motif 4. The heart isoform has 8 fewer amino acids in this region and only 4 direct matches. The extracellular loop between S5 and S6 of Motif 3, where the epitope for a monoclonal antibody to the skeletal muscle isoform (mab IIF7 [13]) is located, also shows significant divergence, possibly explaining the lack of cross-reactivity of this antibody between skeletal muscle and heart (Campbell, K., personal communication). However, the

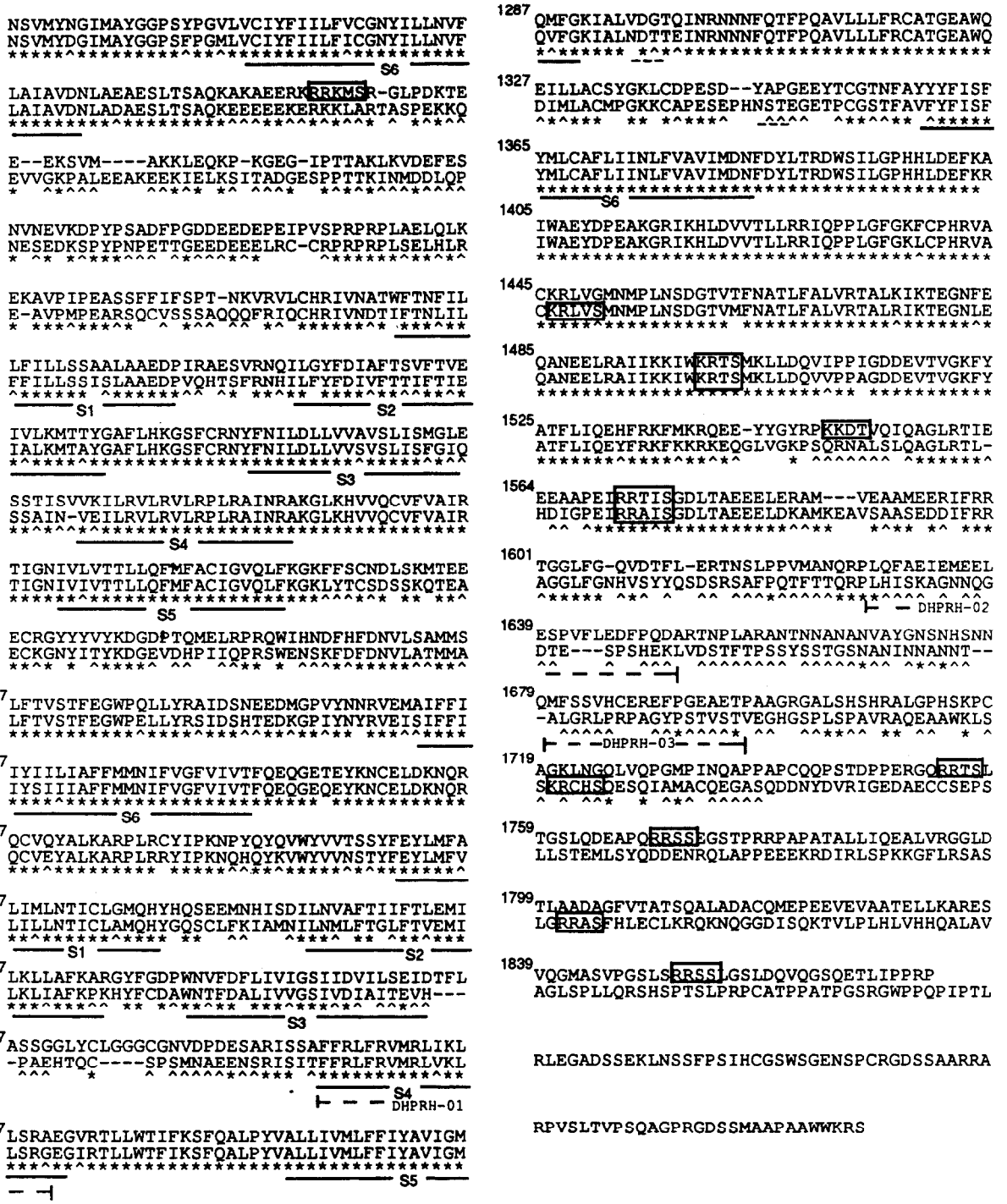

Fig.2. Deduced amino acid sequence of HTDHP 2.0 vs the skeletal muscle α_1. In this figure, the first row of amino acids represents the skeletal muscle α_1 primary structure [12] and the second row represents the sequence deduced from HTDHP 2.0. In the third row, * represents direct matches between the sequences and ∧ represents conservative changes. Boxed in areas are potential PKA phosphorylation sites, and dashes are potentiated N-linked glycosylation sites. Transmembrane segments and segments from which the oligonucleotides for the hybrid arrest experiments were derived are underlined.

Table 1

The effect of DHPRH-oligonucleotide probes on the expression of heart L-type VDCC in *Xenopus* oocytes

RNA and treatment	Currents (nA)						% inhibition of Bay K 8644-sensitive heart current with oligonucleotide probe
	No Bay K 8644			With Bay K 8644			
	Mean	(*n*)	SE (±)	Mean	(*n*)	SE (±)	
None[a]	−8.00	(5)	3.00	–	–	–	–
Rabbit heart[b]	−25.00	(5)	8.00	−96.00	(6)	16.00	–
Rabbit heart[c] DHPRH-O_1 (25 μg/ml)	−4.00	(4)	3.00**	−10.00	(4)	2.00***	89
Rabbit heart[c] DHPRH-O_2 (25 μg/ml)	−6.00	(5)	7.00**	−11.00	(5)	2.00***	88
Rabbit heart[c] DHPRH-O_3 (25 μg/ml)	−6.00	(5)	3.00*	−19.00	(5)	1.00***	80

[a] Oocytes were injected with 50 nl hybridization buffer heated to 65°C for 2 min, the 37°C for 3.5 h
[b] Oocytes were injected with 50 nl rabbit heart RNA solution (7 mg/ml) treated as in 'a'
[c] Oocytes were injected with 50 nl hybridization mixture treated as in 'a'

* Significant, $p < 0.05$; ** significant, $p < 0.025$; *** significant, $p < 0.0005$. The experiments were performed with oocytes of one frog. Entries are mean values; number of cells tested are given in parentheses. Negative values correspond to inward currents. Total RNA from 8-day-old rabbit heart was prepared according to [25]. Oocytes, where indicated, were injected with 50 nl RNA solution (7 mg/ml). Hybridization with oligonucleotide probes was done as described in [20]. Total RNA concentration was 7 mg/ml in all hybridizations and each oligonucleotide probe was at 25 μg/ml. Bay K 8644 (1 μM was previously shown to be ineffective in increasing the endogenous calcium current in oocytes injected with hybridization buffer alone [20]. Microinjection of oligonucleotide probes in hybridization buffer failed to elicit calcium currents significantly higher than those of noninjected oocytes [20]. Oligonucleotide probes: DHPRH-O_1 (5′ CGCGGCTCAG CAGCTTGACC AGGCGCATGA CCCGGAACAG GCGGAAGAAG G3′) is a reverse complement of the HTDHP2.0 corresponding to the putative S4 region in the Motif 4 and represents the region between amino acid residues 1234–1250. DHPRH-O_2 (5′ TGGACGAGTA GCTGCTGGGG GTGAAAGTGG AGTCCACCAG CTTCTCGTGG 3′) is a reverse complement located between amino acid residues 1630–1647. DHPRH-O_3 (5′ GGAGCTGAGC TTCCATGCTG CCTCCTGTGC CCGGACGGCA GGAGACAAGG 3′) is a reverse complement of a stretch between amino acid residues 1677–1694. The numbering of amino acids corresponds to that of fig.2. Statistical analysis was done by using a one-tailed *t*-test

S4 segments, which are proposed to be the voltage sensors in all voltage-dependent cation channels cloned thus far [21,22], also contain positively charged arginines and lysines in every 3rd or 4th position.

The differences in the amino acid sequence result in a number of changes in potential sites for protein kinase A (PKA) phosphorylation. Five of the proposed sites in the skeletal muscle are absent in the heart isoform (serine residues 687, 1757, 1772, and 1854, and threonine residue 1552) and 2 are conserved (serine residues 1502 and 1575). The deduced structure of the partial clone shows 3 new potential PKA sites at serine residues that correspond to 1450, 1724 and 1804 of the skeletal muscle sequence. Two new potential extracellular *N*-glycosylation sites also appear in the heart isoform at residues corresponding to 1295 and 1344.

Northern blots made with heart poly(A^+) RNA were hybridized with different segments of HTDHP 2.0 cDNA (fig.3, lanes 1–3). Each of the probes hybridized with two mRNA species, one at 8.5 kb and another at 13 kb. With each of the probes, the 8.5 kb transcript appeared to provide the prominent band, however, the weaker 13 kb transcript is also detectable. When heart poly(A^+) RNA was hybridized with a skeletal muscle α_1 probe (4.3 kb *Eco*RI/*Sac*I fragment) 2 weak hybridization signals, at 6.5 kb and 8.5 kb (fig.3,

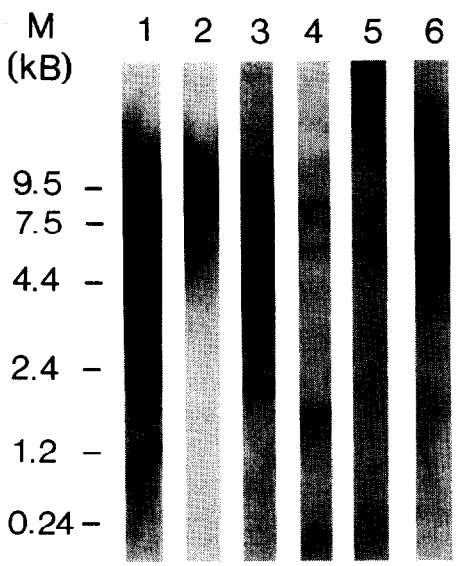

Fig.3. Northern blots of skeletal muscle and heart RNA. Rabbit heart poly(A^+) RNA and skeletal muscle total RNA were fractionated on a formaldehyde-agarose gel and transferred to a Nytran filter. Lanes 1–4 contain rabbit heart poly(A^+) RNA (2 μg each) and lanes 5,6 contain rabbit skeletal muscle total RNA (10 μg each). The blots were probed with fragments of the heart and skeletal muscle cDNAs as follows: lane 1, 1.6 kb *Sma*I fragment of HTDHP1.0; lane 2 and 5, 1.3 kb *Bgl*II fragment of HTDHP1.0; lane 3, 0.9 kb *Bgl*II fragment of HTDHP2.0; lanes 4 and 6, 4.3 kb *Eco*RI/*Sac*I from the skeletal muscle α_1 cDNA. Lanes 1, 2, 3, 5 and 6 were washed in 0.1 × SSPE with 0.1% SDS at 55°C. Lane 4 was washed in 1 × SSPE, 0.1% SDS at 55°C. The 4 kb band in lane 1 and the 1.8 kb band in lane 4 were not reproducible and probably represent artifacts.

lane 4), were noted. In contrast, the 1.3 kb *Bgl*II fragment of clone HTDHP 2.0 only weakly hybridized to a 6.5 kb transcript in skeletal muscle total RNA (fig.3, lane 5). When Northern blots made from skeletal muscle total RNA were hybridized with a skeletal muscle α_1 probe, the prominent transcript was a 6.5 kb message as previously reported [12,13].

4. DISCUSSION

The hybrid arrest of mRNA expression in *Xenopus* oocytes presented here clearly show that oligonucleotides derived from the putative heart specific cDNA are highly effective in inhibiting the expression of L-type VDCC activity in oocytes. As expected, the oligonucleotide which is complementary to the S4 region (DHPRH-O_1) exhibited a high degree (89%) of inhibition; this region is highly conserved among various ion channels [22]. Similar inhibition of current expression by the other 2 probes, which are specific for the HTDHP 2.0 cDNA verifies that this clone codes for a cardiac isoform of the VDCC α_1 subunit. It is important to note that the oligonucleotide probes are probably specific to calcium channel, since an expressed sodium channel was not inhibited.

The deduced amino acid sequence of HTDHP 2.0 shows striking similarity to the skeletal muscle DHP receptor, but significant differences were also identified. Very strong similarities are seen in the areas of the membrane spanning regions. This was expected, as other ion channels also show significant homology in these regions [21,22].

Differences between the heart and skeletal muscle sequences are most apparent in extracellular and intracellular loops and in the cytoplasmic carboxyl tail. Five of the potential PKA phosphorylation sites in the skeletal muscle are absent from the heart sequence. Among these 5 sites is Ser 687, which has been shown to be rapidly phosphorylated by PKA [23] and has been proposed to be the in vivo site of regulation by this kinase [24]. In the heart sequence, however, an Ala is substituted for Ser 687. There is also a substitution for skeletal muscle Ser 1617, a residue that was found to be phosphorylated slowly in vitro [24].

The considerable differences in the sequence between S3 and S4 segments of Motif 4, as well as among other extracellular domains, may contribute to the differences in antigenicity between the heart and skeletal muscle α_1 subunits. Antibodies raised to the α_1 subunit from skeletal muscle show no cross-reactivity with other tissues (Campbell, K., personal communication). The differences in potential glycosylation sites in these segments may contribute to this as well. The differences in sequence when compared to other L-type channel isoforms, in aspects such as structural domains, phosphorylation sites, and glycosylation sites, may lead us to the identification of domains which are important for drug binding and kinetics of channel activation and inactivation.

Northern analysis also shows major differences between the skeletal muscle and heart isoforms. The larger sizes of the heart transcripts indicate that the 2 proteins may be produced by different

genes or by differential splicing of transcripts from the same gene. The former seems more probable, as the nucleotide sequences show differences scattered throughout the sequence rather than large blocks of similar and dissimilar regions (not shown). The sizes of the heart transcripts also suggest that the heart α_1 subunit may be a larger protein than that of skeletal muscle. We showed here that it is 73 amino acids longer at the C-terminus; it may also be significantly longer towards the N-terminus. Alternatively, the transcripts may contain unusually long untranslated regions, although the 3′-untranslated sequence appears to be of normal length.

Acknowledgements: The authors would like to thank Drs David McLennan and Jonathan Lytton for the gift of the λgt10 cDNA library, Walter Koch and Anna Hui for valuable technical advice, and Dr Pal L. Vaghy for critically reading the manuscript. The (−)Bay K 8644 was a gift from Bayer. This work was supported by NIH grants HL07382, HL22619 and HL41496. The oocyte experiments were supported by a grant to N.D. from HDA US-Israel Binational Science Foundation.

REFERENCES

[1] Hess, P., Lansman, J.B. and Tsien, R.W. (1985) Nature 316, 443–446.
[2] Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Nature 316, 440–443.
[3] Yaari, Y., Hamon, B. and Lux, H.D. (1987) Science 235, 680–682.
[4] McCleskey, E.W., Fox, A.P., Feldman, D. and Tsien, R.W. (1986) J. Exp. Biol. 124, 177–190.
[5] Hofmann, F., Nastainczyk, W., Röhrkasten, A., Schneider, T. and Sieber, M. (1987) Trends Pharmacol. Sci. 8, 393–398.
[6] Schwartz, A., Vaghy, P.L. and McKenna, E. (1988) Am. J. Cardiol. 62, 3G–6G.
[7] Schwartz, A. and Triggle, D.J. (1984) Annu. Rev. Med. 35, 325–339.
[8] Ellrodt, A.G. and Singh, B.N. (1983) Pharm. Ther. 23, 1–43.
[9] Vaghy, P.L., McKenna, E., Itagaki, K. and Schwartz, A. (1988) Trends Pharmacol. Sci. 9, 398–402.
[10] Catterall, W.A., Seagar, M.T. and Takahashi, M. (1988) J. Biol. Chem. 263, 3535–3538.
[11] Naito, K., Itagaki, K., McKenna, E., Schwartz, A. and Vaghy, P.L. (1989) Biophys. J. 55, 438a.
[12] Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hilrose, T. and Numa, S. (1987) Nature 328, 313–318.
[13] Ellis, S.B., Williams, M.E., Ways, N.R., Brenner, R., Sharp, A.H., Leung, A.T., Campbell, K.P., McKenna, E., Koch, W.J., Hui, A., Schwartz, A. and Harpold, M.M. (1988) Science 241, 1661–1664.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp.202–203, Cold Spring Harbor, NY.
[15] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[16] Chomczynski, P. and Sacci, N. (1987) Anal. Biochem. 163, 156–159.
[17] Aviv, H. and Lederer, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[18] Saner, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[19] Wernke, G.R. and Thompson, R.L. (1989) Biophys. J. 59, 390a.
[20] Lotan, I., Goelet, P., Gigi, A. and Dascal, N. (1989) Science 243, 666–669.
[21] Jan, L.Y. and Jan, Y.N. (1989) Cell 56, 13–25.
[22] Catterall, W.A. (1988) Science 242, 50–61.
[23] Jahn, H., Nastainczyk, W., Röhrkasten, A., Schneider, T. and Hofmann, F. (1988) Eur. J. Biochem. 179, 535–542.
[24] Röhrkasten, A., Meyer, H., Nastainczyk, W., Sieber, M. and Hofmann, F. (1988) J. Biol. Chem. 263, 15325–15329.
[25] Dierks, P., Van Ooyen, A., Mantei, N. and Weissmann, C. (1981) Proc. Natl. Acad. Sci. USA 78, 1411–1415.