Binding of Ala-scanning analogs of ω-conotoxin MVIIC to N- and P/Q-type calcium channels

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Abstract ω-Conotoxin MVIIC binds to P/Q-type calcium channels with high affinity and N-type channels with low affinity. To reveal the residues essential for subtype selectivity, we synthesized Ala-scanning analogs of MVIIC. Binding assays using rat cerebellar P_2 membranes suggested that Thr^{11}, Tyr^{13} and Lys^2 are essential for binding to both N- and P/Q-type channels, whereas Lys^4 and Arg^{22} are important for binding to P/Q-type channels. These results suggest that MVIIC interacts with P/Q-type channels via a large surface, in good agreement with previous observations using chimeric analogs.

Key words: ω-Conotoxin MVIIC; Calcium channel; Ala-scanning

1. Introduction

Voltage-gated calcium channels play crucial roles in regulating intracellular calcium concentrations in a wide variety of cells and have been classified into several subtypes according to their electrophysiological and pharmacological properties [1–3]. Among them, N- and P/Q-type channels are essential for the regulation of neurotransmitter release from a wide variety of neurons. Various specific peptide ligands have been used for the pharmacological distinction of calcium channel subtypes, including the ω-conotoxins isolated from the venom of marine *Conus* snails. The defining ligands for N-type calcium channels are ω-conotoxin GVIA and MVIIA, while P/Q-type channels are blocked by ω-conotoxin MVIIC (Fig. 1) [4]. Because MVIIC retains weak affinity for N-type channels, it is important to develop more selective blockers for P/Q-type channels. We reported previously that the N-terminal half of MVIIC is important for recognition of N-type channels, whereas essential residues for P/Q-type channel recognition are located on both N- and C-terminal halves based on results with two chimeric analogs of MVIIA and MVIIC [5].

In the present study, a series of analogs of MVIIC were synthesized by replacing each amino acid residue except for Gly, Ala and Cys with Ala in order to identify the essential residues for binding to N- and P/Q-type calcium channels. Results of binding assays suggested that a limited number of residues are essential for binding to N-type channels, whereas the essential residues for recognition of P/Q-type channels are widely spread over the MVIIC molecule, in good agreement with the previous observations using chimeric analogs.

2. Materials and methods

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids, Fmoc-NH-resin and other reagents used on a synthesizer were obtained from Perkin Elmer-Applied Biosystems (Chiba, Japan). Other reagents were obtained from Watanabe Chemical Industries (Hiroshima, Japan), Peptide Institute (Osaka, Japan) or Kokusan Chemical Works (Tokyo, Japan). Lysyl endopeptidase and thermolysin were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Synthesis and purification of peptides

Solid phase peptide synthesis was performed on a Perkin Elmer-Applied Biosystems 431A peptide synthesizer. Amino acid analyses were performed on a Beckman System Gold amino acid analyzer after hydrolysis in 6 M hydrochloric acid at 110°C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulfonyl chloride. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out with a PerSeptive Biosystems Voyager Linear DE mass spectrometer using α-cyano-4-hydroxy-cinnamic acid as a matrix. Analytical high performance liquid chromatography (HPLC) was conducted on a Shimadzu LC-6A system with an ODS column Shim-pack CLC-ODS (4.6×250 nm, Shimadzu). Preparative HPLC was performed on a Shimadzu LC-8A system with an ODS column Shim-pack PREP-ODS (H) (20×250 nm, Shimadzu).

All the analogs were synthesized by a procedure similar to that described previously for the synthesis of MVIIC and its analogs [5,6]. Briefly, linear precursors of MVIIC analogs were synthesized by solid phase methodology of Fmoc chemistry. After trifluoroacetic acid cleavage, crude linear peptide was diluted to the final peptide concentration of 0.05 mM and subjected to oxidative disulfide bond formation at 4°C for 3–5 days in 1 M ammonium acetate buffer (pH 7.8) containing reduced/oxidized glutathione (molar ratio of peptide: GSH:GSSG was 1:100:10). The folding reaction was monitored by

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Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; GSH, reduced glutathione; GSSG, oxidized glutathione; GVIA, ω-conotoxin GVIA; HPLC, high performance liquid chromatography; [^{125}I]GVIA, [^{125}I]ω-conotoxin GVIA; [^{125}I]MVIIC, [^{125}I][Nle^{12}]ω-conotoxin MVIIC; MALDI-TOF-MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MVIIA, ω-conotoxin MVIIA; MVIIC, ω-conotoxin MVIIC; ODS, octadecylsilane; TFA, trifluoroacetic acid; Analogs are designated by a letter and number indicating the identity and position of the substituted amino acid, followed by a letter indicating the identity of the replacement residue; for example, **K2A** indicates an analog in which Lys^2 is replaced with Ala

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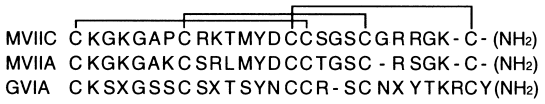

Fig. 1. Amino acid sequences and disulfide bonds of ω-conotoxin MVIIC, MVIIA and GVIA.

HPLC and stopped by lowering the pH of the solution to 3–4 with AcOH. The crude cyclic products were purified by successive chromatography on Sephadex G-50F, CM-cellulose CM-52 and preparative HPLC with an ODS column. The structure and purity of synthetic peptides were confirmed by analytical HPLC, amino acid analysis and MALDI-TOF-MS measurements.

2.3. Enzymatic digestion for the determination of disulfide bond combination

To a solution of synthetic peptide (0.4 mg) in 100 μl of 0.1 mM phosphate buffer (pH 6.5) was added a solution of lysyl endopeptidase (10 μg) in 20 μl of the same buffer. The mixture was incubated at 37°C for 1.5 h and subjected to HPLC separation and MALDI-TOF-MS measurements. The major fragment was lyophilized and dissolved into 0.4 ml of 0.1 M ammonium formate buffer (pH 6.5). To 100 μl of this solution were added a solution of thermolysin (20 μg) in 20 μl of the same buffer and 80 μl of $CaCl_2$ solution (2.5 mM in the same buffer). The mixture was incubated at 37°C for 3 h and subjected to HPLC separation and MALDI-TOF-MS measurements.

2.4. CD measurements

CD spectra were recorded on a JASCO J-600 spectropolarimeter in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C, using a quartz cell of 1 mm path length. The spectra are expressed as molar ellipticity (θ).

2.5. Binding assay

Rat cerebellar P_2 membranes (10 μg) in 0.1 ml of 25 mM Tris, 150 mM NaCl, 0.1% bovine serum albumin adjusted to pH 7.4 with HCl (TBSA) were incubated with 0.5 nM [^{125}I]GVIA or [^{125}I]MVIIC for 1 h at 30°C. Membrane-bound radioactivity was measured after rapid filtration and washing on GF/C (Whatman) filters treated with 0.3% polyethyleneimine as described previously [7].

3. Results

3.1. Synthesis and purification of MVIIC analogs

All the linear precursors of MVIIC analogs were successfully assembled by solid phase methodology. Air oxidation of the crude linear precursors of most analogs afforded peptides with proper disulfide bondings as the major products, with good overall isolation yields. However, cyclization of two analogs, **P7A** and **D14A**, in which Pro^7 and Asp^{14} were replaced with Ala, respectively, gave mixtures of several products with a variety of retention times in HPLC analysis (Fig. 2).

3.2. Disulfide bond combination

According to the method described for MVIIC by Kubo et al. [8], we successively digested most analogs with lysyl endopeptidase and thermolysin. Fig. 3 shows the cleavage sites of

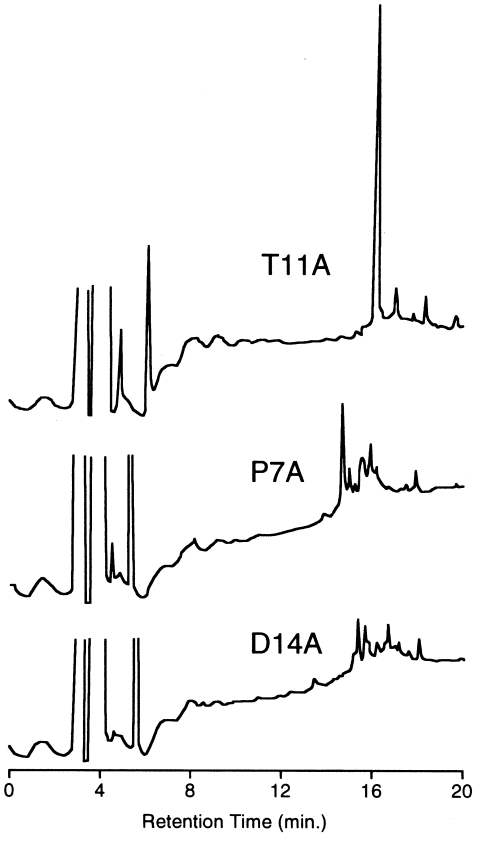

Fig. 2. HPLC profiles of the oxidative folding reaction of MVIIC analogs. Each chromatogram shows the HPLC profile of reaction mixture after 3 days. Large peaks at around 4 min are due to the solvent, GSH or GSSG. Column: Shim-pack CLC-ODS (4.6×250 mm, Shimadzu). Solvent: linear gradient from 5 to 35% CH_3CN in 0.1% TFA for 30 min. Flow rate: 1 ml/min. Monitoring: absorbance at 230 nm (intensity is not scaled).

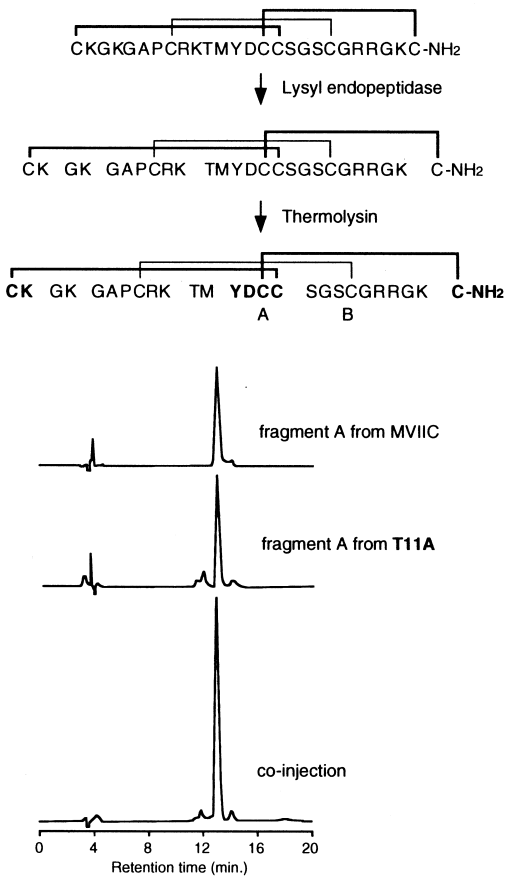

Fig. 3. Scheme of the enzymatic digestion of MVIIC and HPLC profiles of fragment A derived from MVIIC and **T11A**. Same conditions as in Fig. 2 except for isocratic elution with 3% CH_3CN in 0.1% TFA.

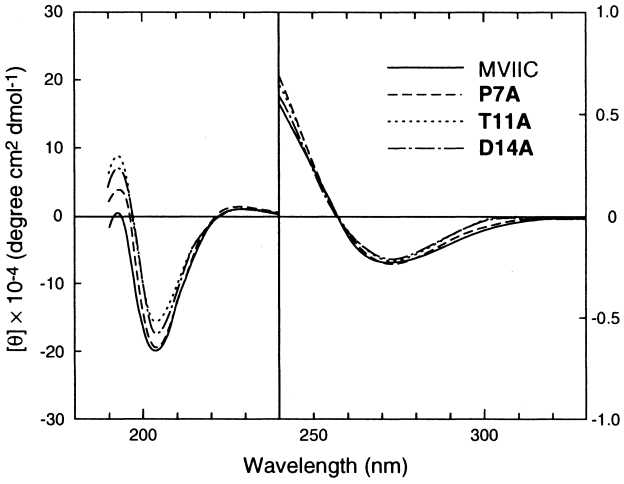

Fig. 4. CD spectra of MVIIC and its analogs in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C.

the peptide bonds in MVIIC and the HPLC comparison of digestion products of MVIIC and **T11A**. The result indicated that **T11A**, the weakest analog (described below), has the same disulfide bond pairings as native MVIIC. All the analogs except for **K2A**, **Y13A**, **D14A**, **S17A** and **K25A** were digested and gave the same fragment A as native MVIIC.

3.3. CD spectra

CD spectra of all the analogs including **K2A**, **Y13A**, **D14A**, **S17A** and **K25A** were similar to that of MVIIC with positive Cotton Effects around 230 nm and negative ones around 203 nm, suggesting that the conformations of the analogs are similar to that of MVIIC. Certain CD spectra are illustrated in Fig. 4 as examples.

3.4. Biological activity

The ability of the analogs to inhibit binding of [^{125}I]GVIA and [^{125}I]MVIIC to rat cerebellar P_2 membranes were compared to displacement by MVIIA and MVIIC. The IC_{50} values of the analogs are summarized in Fig. 5. We have previ-

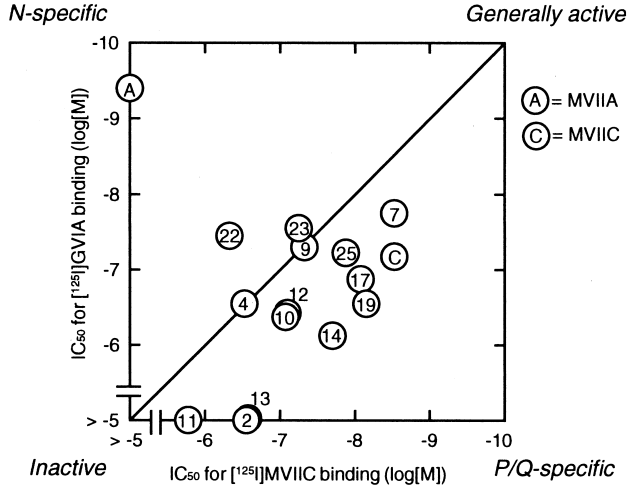

Fig. 5. Inhibition (IC_{50}) by MVIIC analogs of [^{125}I]GVIA or [^{125}I]MVIIC binding to rat cerebellar P_2 membranes. Numbers in circles indicate the residues replaced with Ala. Letters A and C in circles indicate the IC_{50} of native MVIIA and MVIIC, respectively.

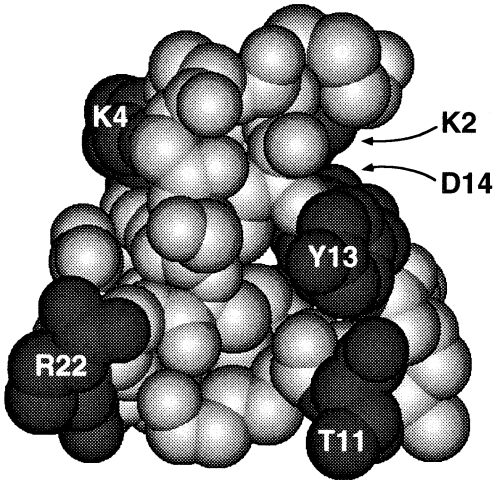

Fig. 6. Location of certain residues essential for the activity or oxidative folding reaction. The model was drawn by using the cord 1OMN of the Protein Data Bank [13].

ously reported that Tyr^{13} is important for the binding of MVIIC to P/Q-type calcium channels [6]. For binding to P/Q-type channels, substitution at several other residues resulted in a significant loss of affinity. Among these, Thr^{11} was the most marked with an increase in the IC_{50} value of the Ala substituted analog to almost 500 times that of MVIIC. Lys^2, Lys^4 and Arg^{22} were also implicated, suggesting that ionic interaction is important for binding. For the binding to N-type channels, only three residues, Lys^2, Thr^{11} and Tyr^{13}, were essential.

4. Discussion

Oxidative disulfide bond formation is a key step for the synthesis of peptides with multiple disulfide bonds. Hyp residues were essential for the folding of μ-conotoxin GIIIA due to its cyclic structure [9] and Asn^{20} and Thr^{23} residues for ω-conotoxin GVIA probably due to intramolecular hydrogen bonds involving their side chains [10,11]. In the present study, replacement of Asp^{14} in MVIIC had the most significant effect on the folding reaction, as suggested by Fig. 2. Because Asp^{14} is close to Lys^2 in the three dimensional structure [12,13], ionic interaction may be important for the folding in the case of MVIIC (Fig. 6).

For the structure–activity relationship study of peptides with multiple disulfide bonds, it is essential to confirm that the analogs have the same disulfide pairings as the native peptide. In most analogs, a comparison of their enzymatic digestion products to that of native MVIIC clearly showed that they possess the same disulfide pairings as native MVIIC. However, this method was not applied to analogs **K2A**, **Y13A**, **D14A**, **S17A** and **K25A**, in which replacement might affect either enzyme specificity or the structure of digestion products. Therefore, we measured the CD spectra of the analogs to confirm indirectly that the analogs had a similar conformation to MVIIC. All the analogs showed the similar CD spectra to that of MVIIC, suggesting that they have similar conformation including common disulfide bond pairings.

The three-dimensional structures of GVIA [14–17], MVIIA [18,19] and MVIIC [12,13] have been determined by NMR analysis. Despite differences in primary amino acid sequences,

the polypeptide chain framework is conserved in all of the ω-conotoxins. Thus the nature of the amino acid side chains may have a dominant role in determining the toxin selectivity.

Previously, we showed that Tyr^{13} is essential for the activity of GVIA and that Lys^2 is the second most important residue, based on the results of systematic single Ala substitution [20,21]. Because replacement of other residues by Ala did not affect binding, we proposed a two-point binding model between GVIA and the N-type calcium channel [20]. Replacement of Tyr^{13} of MVIIA by Ala also resulted in a significant loss of affinity, whereas substitution of Lys^2 did not affect the binding [22]. Loss of the basic side chain of Lys^2 may be compensated by other basic residues such as Lys^7 [18], which is substituted by a neutral amino acid in GVIA and MVIIC. Nadasdi et al. also reported that the replacement of Tyr^{13} in MVIIA by Phe reduced affinity to 0.5% of that of native MVIIA and that the elimination of certain positive charges also perturbed binding [23]. These previous observations are in good agreement with the present results that only three residues in MVIIC, Lys^2, Thr^{11} and Tyr^{13}, were essential for binding to N-type channels. These three residues are located on the same side of MVII molecule (Fig. 6). It will be interesting to further explore the role of Thr^{11} of MVIIC, since N-type specific MVIIA and GVIA have Leu and Thr residues at the 11th position, respectively.

For the binding of MVIIC to P/Q-type channels, we previously reported that the replacement of Tyr^{13} by Ala significantly reduced the affinity, suggesting that Tyr^{13} is a common binding motif in ω-conotoxins irrespective of the calcium channel subtypes that they target [6]. However, replacement of Thr^{11} showed the largest reduction of affinity as shown in Fig. 5. Furthermore, the replacement of many other residues also resulted in a large reduction in affinity. Consequently, the results of single alanine substitutions of MVIIC agreed well with the prediction that MVIIC has a broad binding surface with P/Q-type calcium channels, based on the data from chimeric analogs (Fig. 6). To develop more P/Q specific conotoxins, it will be necessary to examine combined effects of simultaneous multiple substitutions.

Voltage-gated calcium channels are complex membrane proteins consisting of multiple subunits [1–3]. A central channel pore is formed by an α_1 subunit that has four motifs, I–IV, each having six transmembrane segments, S1–S6. A pore-lining segment H5 between segments 5 (S5) and 6 (S6) is thought to be essential for the ion selectivity. The α_1 subunits of N- and P/Q-type calcium channels have been cloned and designated as α_{1B} and α_{1A}, respectively, according to the nomenclature of voltage-gated calcium channels [24]. The amino acid sequences of H5 segments are almost identical between N- and P/Q-type calcium channels [25,26]. Cloning, mutagenesis and expression of α_1 subunits showed that the most dramatic effects on the interaction between GVIA and N-type calcium channel involved at a single cluster of residues in the large extracellular loop between IIIS5 and IIIH5, consistent with a direct pore-blocking mechanism [27]. A combination of mutational studies on both ion channels and their specific blockers will provide knowledge of the architecture of the outer vestibules of the channel pores. In combination with the comparison of amino acid sequences of H5 segments of calcium channels, studies on the blocking mechanism of ω-conotoxins may enable the design and synthesis of novel blockers with appropriate specificities.

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References

[1] Tsien, R.W., Ellinor, P.T. and Horne, W.A. (1991) Trends Pharmacol. Sci. 12, 349–354.
[2] Miller, R.J. (1992) J. Biol. Chem. 267, 1403–1406.
[3] Randall, A. and Tsien, R.W. (1995) J. Neurosci. 15, 2995–3012.
[4] Olivera, B.M., Miljanich, G.P., Ramachandran, J. and Adams, M.E. (1994) Ann. Rev. Biochem. 63, 823–867.
[5] Sato, K., Raymond, C., Martin-Moutot, N., Sasaki, T., Omori, A., Ohtake, A., Kim, J.-I., Kohno, T., Takahashi, M. and Seager, M.J. (1997) FEBS Lett. 414, 480–484.
[6] Kim, J.-I., Takahashi, M., Martin-Moutot, N., Seagar, M.J., Ohtake, A. and Sato, K. (1995) Biochem. Biophys. Res. Commun. 214, 305–309.
[7] Martin-Moutot, N., Leveque, C., Sato, K., Kato, R., Takahashi, M. and Seagar, M. (1995) FEBS Lett. 366, 21–25.
[8] Kubo, S., Chino, N., Kimura, T. and Sakakibara, S. (1996) Biopolymers 38, 733–744.
[9] Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., Nakamura, H., Ohya, M., Lancelin, J.-M., Kohda, D. and Inagaki, F. (1991) J. Biol. Chem. 266, 16989–16991.
[10] Kim, J.-I., Takahashi, M., Ogura, A., Kohno, T., Kudo, Y. and Sato, K. (1995) in: Peptides 1994 (Maia, H.L.S., Ed.) pp 135–136, ESCOM Science Publishers B.V., Leiden.
[11] Lew, M.J., Flinn, J.P., Pallaghy, P.K., Murphy, R., Whorlow, S.L., Wright, C.E., Norton, R.S. and Angus, J.A. (1997) J. Biol. Chem. 272, 12014–12023.
[12] Nemoto, N., Kubo, S., Yoshida, T., Chino, N., Kimura, T., Sakakibara, S., Kyogoku, Y. and Kobayashi, Y. (1995) Biochem. Biophys. Res. Commun. 207, 695–700.
[13] Farr-Jones, S., Miljanich, G.P., Nadasdi, L., Ramachandran, J. and Basus, V.J. (1995) J. Mol. Biol. 248, 106–124.
[14] Sevilla, P., Bruix, M., Santoro, J., Gago, F., Garcia, A.G. and Rico, M. (1993) Biochem. Biophys. Res. Commun. 192, 1238–1244.
[15] Davis, J.H., Bradley, E.K., Miljanich, G.P., Nadasdi, L., Ramachandran, J. and Basus, V.J. (1993) Biochemistry 32, 7396–7405.
[16] Pallaghy, P.K., Duggan, B.M., Pennington, M.W. and Norton, R.S. (1993) J. Mol. Biol. 234, 405–420.
[17] Skalicky, J.J., Metzler, W.J., Ciesla, D.J., Galdes, A. and Pardi, A. (1993) Protein Sci. 2, 1591–1603.
[18] Kohno, T., Kim, J.-I., Kobayashi, K., Kodera, Y., Maeda, T. and Sato, K. (1995) Biochemistry 34, 10256–10265.
[19] Basus, V.J., Nadasdi, L., Ramachandran, J. and Miljanich, G.P. (1995) FEBS Lett. 370, 163–169.
[20] Sato, K., Park, N.-G., Kohno, T., Maeda, T., Kim, J.-I., Kato, R. and Takahashi, M. (1993) Biochem. Biophys. Res. Commun. 194, 1292–1296.
[21] Kim, J.-I., Takahashi, M., Ogura, A., Kohno, T., Kudo, Y. and Sato, K. (1994) J. Biol. Chem. 269, 23876–23878.
[22] Kim, J.-I., Takahashi, M., Ohtake, A., Wakamiya, A. and Sato, K. (1995) Biochem. Biophys. Res. Commun. 206, 449–454.
[23] Nadasdi, L., Yamashiro, D., Chung, D., Tarczy-Hornoch, K., Adriaenssens, P. and Ramachandran, J. (1995) Biochemistry 34, 8076–8081.
[24] Birnbaumer, L., Campbell, K.P., Catterall, W.A., Harpold, M.M., Hofman, F., Horne, W.A., Y., M., Schwartz, A., Snutch, T.P., Tanabe, T. and Tsien, R.W. (1994) Neuron 13, 505–506.
[25] Williams, M.E., Brust, P.F., Feldman, D.H., Patthi, S., Simerson, S., Maroufi, A., McCue, A.F., Veliçelebi, G., Ellis, S.B. and Harpold, M.M. (1992) Science 257, 389–395.
[26] Mori, Y., Friedrich, T., Kim, M.S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofman, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. and Numa, S. (1991) Nature 350, 398–402.
[27] Ellinor, P.T., Zang, J.F., Horne, W.A. and Tsien, R.W. (1994) Nature 372, 272–275.