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Electrophysiological activity of the C-peptide of the *Locusta* insulin-related peptide

Effect on the membrane conductance of *Locusta* neurones in vitro

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The C-peptide of *Locusta* insulin-related peptide, which is a 50 residue peptide originally isolated from the corpora cardiaca of the insect *Locusta migratoria* and to which we refer as 5-kDa peptide, has been synthesised chemically by the solid-phase method, using a BOC strategy. Since this peptide contains in its sequence a potential monobasic cleavage site, we also synthesised its 1–38 residue-related fragment, named 4-kDa peptide, although we have no hints of its natural occurrence in the corpora cardiaca. Electrophysiological studies have shown that both the 5-kDa and 4-kDa peptides depolarise the membrane and increase the membrane conductance of neurones freshly isolated from the thoracic ganglia of *Locusta*. Under voltage-clamp conditions, the current underlying these effects was inwardly directed and could be resolved into 2 components. One component, $I(5\text{-kDa})_1$, activated at potentials more hyperpolarised than −50 mV, peaked at about −75 mV and was blocked by the potassium channel blockers cesium and rubidium. The second component, $I(5\text{-kDa})_2$ was activated at potentials more depolarised than −50 mV, increased with depolarisation and was not blocked by cesium and rubidium. The effects of the 5-kDa and 4-kDa peptides on the membrane potential and membrane conductance of *Locusta* neurones suggest that these peptides may have a physiological role in the central nervous system of insects.

Neuropeptide; Ionic current; Insulin peptide; C-Peptide

1. INTRODUCTION

Recently, we reported the isolation and structure elucidation of a 5-kDa peptide from the neurohaemal lobes of the corpora cardiaca of the insect *Locusta migratoria* [1]. Comparison of its sequence with protein data banks revealed no significant similarity with other known peptides. This 50-residue neuropeptide is remarkable by its high content in alanine residues (25%) and the presence of a stretch of 5 consecutive alanines. Isolation and nucleotide sequencing of cDNAs obtained using 5-kDa peptide-oligonucleotide probes led to the discovery of a novel member of the superfamily of insulin, namely the *Locusta* insulin-related peptide [2]. In the cDNAs, the sequence encoding for the 5-kDa peptide is flanked by regions homologous to the A and B chains of the insulins of vertebrates [2]. Therefore, the 5-kDa peptide may correspond to the C-peptide or connecting peptide of the *Locusta* insulin-related peptide, although it does not share any significant amino acid sequence homology with the C-peptides of the insulins; this is not surprising, considering that the sequences of the C-peptides have not been well conserved throughout evolution.

In an attempt to find out the possible physiological actions of the 5-kDa peptide we have investigated its effects on neurones isolated from the thoracic ganglia of adult *Locusta*. Since this type of study requires large quantitities of peptide, we have synthesised the 5-kDa peptide chemically using the solid-phase method. We have also synthesised a 4-kDa fragment of the 5-kDa peptide. In the present paper, we show that the synthetic 5-kDa and its 4-kDa related fragment affect the membrane potential and membrane conductance of *Locusta* neurons in vitro, thus suggesting that they may have a physiological function in the central nervous system of this insect. This constitutes, to our knowledge, the first report about a biological effect for a C-peptide of insulin, since, in spite of many attempts, no role beyond correct disulfide bond formation in pro-insulin has been ascribed to the C-peptides of insulins.

Abbreviations: Boc, *N*-tert-butyloxycarbonyl; BOP, benzotriazoyl-*N*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DMF; dimethylformamide; DIEA, *N*-ethyl-diisopropylamin; HF, hydrogen fluoride; NMP, 1-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; CNS, central nervous system; Cs, cesium; Rb, rubidium.

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2. MATERIALS AND METHODS

2.1. *Synthesis*

5-kDa and 4-kDa peptides were synthesised using the solid-phase method of Merrifield [3]. The syntheses were performed manually in

a glass reaction vessel, as described by Plaue and Briand [4]. Amino acids were coupled as Boc derivatives (Neosystem Laboratories). Side chain protecting groups were used as follows: benzyl, (Ser,Thr), *p*-toluenesulfonyl (Arg), 2,6-dichlorobenzyl (Tyr), cyclohexyl (Glu, Asp), formyl (Trp) and xanthenyl (Asn). BocVal-PAM resin (0.2 mM) and Boc-Ala-PAM resin (0.2 mM) (Applied Biosystems) were used as starting material for the 5-kDa and 4-kDa peptides, respectively. Resins were allowed to swell in DCM for 5 min and then washed three times with DCM. The assembly of the peptide chain was then carried out using the procedure summarised in Table I. Briefly, BOP (Neosystem Laboratories) was used as the coupling agent [5], and Boc amino acids were added dissolved in 25% DMF/DCM (v/v). After 15 min coupling, NMP was added to the reaction vessel. Levels of residual free amino groups on the resin were assessed using a qualitative ninhydrin test [6]. When necessary a double or triple coupling was performed and, if the coupling were still incomplete, the unreacted chains were blocked irreversibly by acetylation. To remove the formyl protecting group of the Trp residue, peptide resins were treated with piperidine in DMF. The standard HF procedure was then carried out to remove the other side chain protecting groups and to cleave the peptides from the resins.

2.2. *Purification of synthetic peptides*

The crude synthetic peptides were dissolved in 90% 10% formic acid/water and prepurified on a Fractogel TSK HW-40 (F) (Merck) gel permeation column that had been equilibrated in 10% acetic acid. Fractions containing the required peptides were pooled, lyophilized overnight, redissolved in 0.1% TFA and purified by reverse-phase HPLC on a Waters liquid chromatograph. Absorbance was monitored at 225 nm. A C_8 Aquapore column (0.7 × 25 cm), packed with 7 μm wide pore (30 nm) particles, was used. Elution was performed at a flow rate of 2 ml/min, using a stepwise gradient over 44 min from 15% CH_3CN/0.1% TFA–60% CH_3CN/0.1% TFA. Semi-preparative runs, 2 mg per run, yielded pure 5-kDa and 4-kDa peptides. Finally, fractions containing pure peptides were pooled, checked for purity (purity ca. 98%) on a C18 Vydac column (0.46 × 25 cm) and lyophilised.

2.3. *Amino acid analysis*

5-kDa and 4-kDa peptides (approx. 20 nM) were hydrolysed in constant-boiling 6 M HCl, under argon atmosphere, at 120°C for 24 h. After hydrolysis, amino acids were converted to their phenylthiocarbamoyl derivatives using phenylthioisocyanate as described by the Waters Picotag manual. The derivatives were then identified by reversed-phase HPLC on a Merck Supersher column (0.4 × 11.9 cm) at 45°C, using a gradient in the range 6–60% acetonitrile.

2.4. *FAB mass spectrometry*

FAB mass spectrometry was carried out in the positive mode, using a VG Analytical ZAB-SEQ instrument, at the Service Central d'Analyse, CNRS, Vernaison (France).

2.5. *Electrophysiology*

Experiments were performed on freshly dissociated neuronal somata prepared from the thoracic ganglia of adult *Locusta migratoria* [7]. The isolated cell bodies were maintained for periods of 2–8 h in physiological saline of the following composition: NaCl 180 mM, KCl 10 mM, $CaCl_2$, 10 mM, $MgCl_2$ 15 mM, HEPES 10 mM, pH 6.8.

Conventional single-electrode techniques were employed to obtain current- or voltage-clamp recordings. Thin-walled glass intracellular microelectrodes, of 10–15 MΩ resistance, were back-filled with 1 M KCl solution. 5-kDa and 4-kDa (10^{-5} M to 10^{-4} M) were applied to the cells by pressure ejection from a micropipette positioned 5–10 μm from the impaled somata. To evaluate the effect of the pressure-applied peptides on membrane potential and membrane resistance, hyperpolarising rectangular pulses of current were injected into the impaled neuron during current-clamp recordings. To obtain current-voltage (I–V) curves, the neuronal somata were voltage-clamped at different voltages via a series of 10 mV steps, held at each potential until the membrane current reached a steady level and then challenged with a pressure pulse of 5-kDa or 4-kDa peptides. The action of potassium channel blockers ont he 5-kDa peptide evoked responses was investigated by determining the effect of bath-perfusing the impaled cells with physiological saline containing either 10 mM cesium or 5 mM rubidium. In these experiments, the potassium channel blockers were added to the bathing saline from aqueous 1 M stock solutions. Experiments were performed at room temperature (22–24°C).

3. RESULTS AND DISCUSSION

The 5-kDa peptide was synthesised manually by a solid-phase method, using a BOC strategy and with BOP as the coupling agent. This strategy, together with a gel permeation/reversed-phase HPLC peptide purification procedure, allowed us to obtain an overall yield of approximately 7%. The purity of the final material was shown to be approximately 98%. The identity of the pure synthetic material, assessed by amino acid analysis, showed excellent agreement with the expected one. FAB mass spectrometry on the synthetic 5-kDa peptide provided identical results to those obtained on the natural 5-kDa peptide [1].

The effects of the synthetic 5-kDa peptide were studied in about 60 neurones. Pressure application of 5-kDa (10^{-5} M; 500 ms) onto neurones voltage-clamped at resting potentials (approximately −50 mV) evoked an inward current. The threshold for the response was determined by applying 5-kDa to the cells by bath-perfusion. Bath applications of 5-kDa resulted in dose-dependent increases in inward current with a threshold of about 10^{-8} M (Fig. 1A).

To characterise the voltage-dependence of the current elicited by the 5-kDa peptide, the neurones were voltage-clamped at a series of holding potentials and the amplitude of the 5-kDa-evoked current measured. As illustrated in Fig. $1B_i$ the voltage-dependence of the current evoked by the 5-kDa peptide exhibited a complex voltage-dependence, first decreasing in amplitude between −40 to −50 mV and then increasing in amplitude with hyperpolarisation, reaching a peak at around −70 mV. This complex current-voltage behaviour resulted from the activation of 2 distinct inward currents, $I(5\text{- kDa})_1$ and $I(5\text{-kDa})_2$. These 2 currents could be distinguished on the basis of their voltage-dependence, sensitivity to potassium channel blockers and kinetics (Fig. $1B_{ii}$ and $1B_{iii}$). Furthermore, many neurones tested expressed only one type of 5-kDa-evoked inward current (Figs. 2 and 3).

The first type of current, $I(5\text{-kDa})_1$, activated at potentials more hyperpolarised than −50 mV and peaked at −75 mV, giving a U-shaped I–V curve (Fig. 2B,C). $I(5\text{-kDa})_1$ was inhibited by the potassium channel blockers, Cs and Rb. As illustrated in Fig. $1B_{ii}$ and $1B_{iii}$, exposure of the preparation to physiological saline containing either 5 mM Rb or 10 mM Cs completely blocked $I(5\text{-kDa})_1$. These results suggest that a potassium

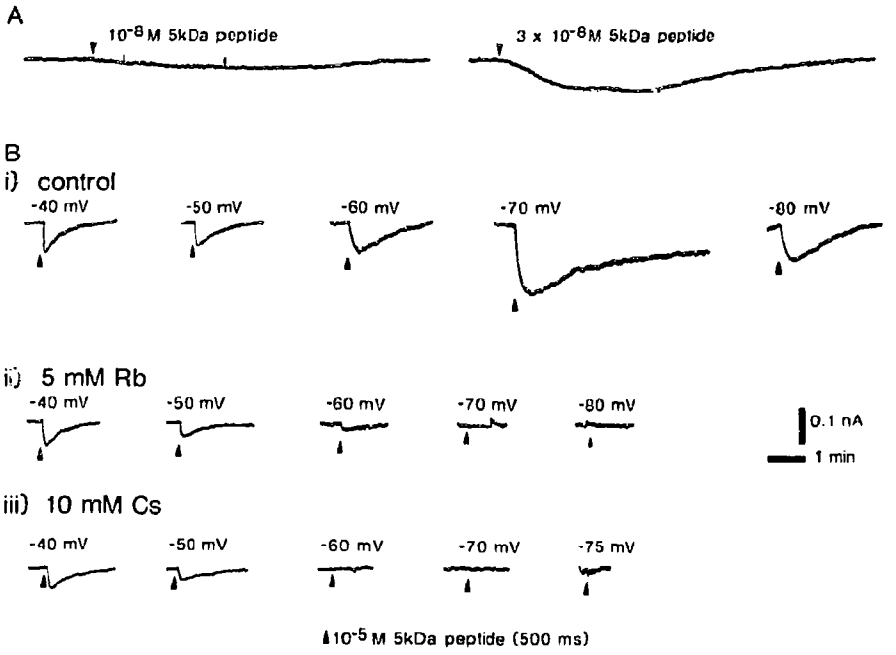

Fig. 1. 5-kDa-evoked responses in neurones freshly isolated from the thoracic ganglia of *Locusta migratoria*: (A) Effect of increasing concentrations of the 5-kDa peptide on the membrane current of *Locusta* neurones voltage-clamped at resting membrane potential (−50 mV). The peptide was bath-perfused for about 2 min (beginning of bath-perfusion is indicated with arrows). Downward deflections represent inward current: (B) Voltage-dependence and ionic mechanism of the 5-kDa-induced current in *Locusta* neurones; (i) The currents induced by 5-kDa peptide were recorded at a series of holding potentials under voltage-clamp conditions. Note the complex voltage-dependence of the current; (ii) 5 mM rubidium (Rb) abolished the currents evoked at potentials more hyperpolarised than −50 mV ($I(5\text{-kDa})_1$) but only decreased slightly the currents evoked at potentials positive to −50 mV ($I(5\text{-kDa})_2$); (iii) 10 mM cesium (Cs) blocked only the current activated at potentials more hyperpolarised than −50 mV ($I(5\text{-kDa})_1$). The current activated at potentials positive to −50 mV ($I(5\text{-kDa})_2$) was only partially inhibited by Rb.

current may be an important component of $I(5\text{-kDa})_1$. Current-clamp analysis of I(5-kDa)1, showed that $I(5\text{-kDa})_1$ was accompanied by a depolarisation and a decrease in membrane resistance as indicated by a decrease in the amplitude of the electronic potentials resulting from hyperpolarising current pulses (Fig. 2A). These results indicate that the 5-kDa peptide opens ion channels. In view of the sensitivity of $I(5\text{-kDa})_1$ to potassium channel blockers it is likely that at least some of the channels activated by the 5-kDa peptide are permeable to potassium ions. Typically, $I(5\text{-kDa})_1$ had a slow time-course, the 5-kDa-evoked inward current reaching its peak in about 20–30 s and the membrane current returning to baseline values in 1–2 min (Fig. 2).

$I(5\text{-kDa})_2$ activated at potentials more depolarised than −50 mV (Fig. 3B) and its time-course was faster than that of $I(5\text{-kDa})_1$. $I(5\text{-kDa})_2$ was also accompanied by a depolarisation and decrease in membrane resistance (Fig. 3A). As shown in Fig. $1B_{ii}$ and $1B_{iii}$, $I(5\text{-kDa})_2$ was only slightly decreased in the presence of the potassium channel blockers Rb and Cs, thus strongly suggesting that potassium ions are not the predominant charge carrier of $I(5\text{-kDa})_2$. Although the ionic mechanism of $I(5\text{-kDa})_2$ has not been fully determined, it is worth noting that $I(5\text{-kDa})_2$ resembles an inward current evoked by the invertebrate neuropeptide, FMRFamide, in *Aplysia* neuron R14. This FMRFamide-evoked current is slow, voltage-dependent, being largest between −40 to −20 mV, and is sodium-dependent [8].

Since the 5-kDa peptide contains in its sequence a potential monobasic cleavage site at the level of residues 39–40 (Arg-Pro), we decided to synthesise its 1–38 related fragment. Indeed, pro-peptide processing occurs most often at pairs of basic amino acids such as Lys-Arg, Arg-Lys and Lys-Lys [9,10]. Although monobasic processing signals are uncommon, several examples have been reported in which precursor cleavage occurs at a single Arg residue [11–13]. In approximately one third of the cases in which a single basic residue is the signal for proteolytic cleavage, a proline residue is found either just before or just after the basic residue [14]. Therefore, although we have no hints of its natural occurrence in the corpora cardiaca, we synthesised the 1–38 fragment of the 5-kDa peptide to test its potential activity. This compound is referred to as the 4-kDa peptide.

The synthesis and purification procedures used to obtain the 4-kDa peptide produced an overall yield of about 5%. The observed molecular mass of the 4-kDa fragment corresponded strictly to the calculated one (observed M_r=3899.37; calculated M_r=3899.03). Pressure application of the 4-kDa peptide onto the impaled cells elicited responses that were identical to those evoked by the 5-kDa peptide, thus indicating that this

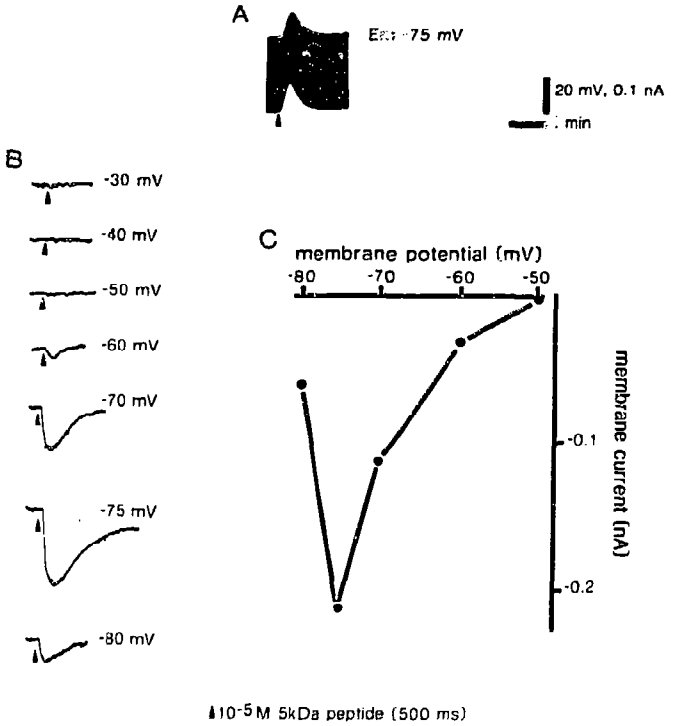

Fig. 2. I(5-kDa)$_1$-evoked response of locust thoracic neurones in vitro: (A) In current-clamped neurones, I(5-kDa)$_1$ resulted, as illustrated, in a depolarisation and decrease in membrane resistance as indicated in this neurone by a decrease in the potential change in response to the repetitive intracellular application of constant, hyperpolarising current pulses: (B) In voltage-clamp experiments, I(5-kDa)$_1$ was, as shown, inwardly directed and activated at potentials negative to −50 mV, reaching a peak at −75 mV: (C) The current-voltage relationship of the 5-kDa-evoked currents shown in (B).

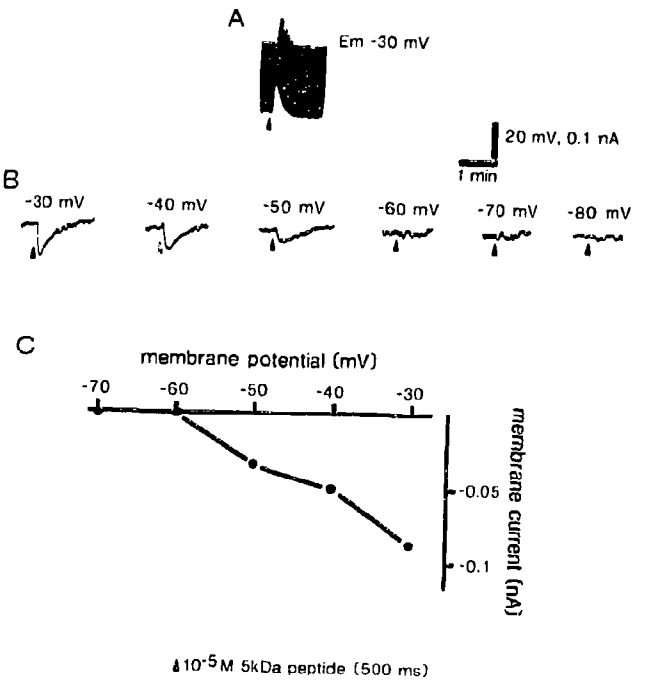

Fig. 3. I(5-kDa)$_2$ response of locust neurones in vitro: (A) In neurons current-clamped at −30 to −40 mV, I(5-kDa)$_2$ resulted in a depolarisation and decrease in membrane resistance as indicated by a reduction in the potential change in response to the intracellular application of hyperpolarising current pulses: (B) Voltage-dependence of I(5-kDa)$_2$. I(5-kDa)$_2$ activated, as illustrated, only at potentials more depolarised than −50 mV: (C) Current-voltage relationship of the I(5-kDa)$_2$ response shown in (B).

peptide is an agonist of the 5-kDa receptor (data not shown). The threshold for this peptide was also between $1–3 \times 10^{-8}$ M.

Our results show 2 independent actions of the peptide 5-kDa and its 1–38 synthetic fragment, the 4-kDa peptide. Each action is associated with a different voltage-dependence and ionic mechanism, thus indicating that they are mediated by different receptors. This multiplicity of receptors and actions are typical of the action of neuropeptides in the CNS of invertebrates (see e.g. [8]). The slow kinetics of the currents elicited by the 5-kDa peptide suggests the involvement of second messengers. Typically, second messenger systems link neuropeptide receptors to voltage-dependent channels regulating the excitability of neurones (see e.g. [15]). The mode of action of the C-peptide of *Locusta* insulin-related peptide is, therefore, similar to those caused by established neuropeptides.

Although the response evoked by the 5-kDa peptide and its agonist, the 4-kDa peptide, is complex, resulting from the activation of 2 distinct inward currents, the overall effect of these peptides consists of ion channel opening. The activation of these channels depolarises the membrane potential of the locust neurones. Therefore, it is possible that the 5-kDa peptide, the C-peptide of the *Locusta* insulin-related peptide, may contribute to the regulation of the excitability of locust central neurones. This is remarkable since the C-peptide of the insulins has been thought, up to now, to be involved mainly in the proper folding of insulin during its processing from pro-insulin to mature insulin, and was considered to be devoid of any biological role, although it has been shown to be released into the circulation [16] and be present in human brain neurones [17]. It will be

Table I

General procedure for peptide chain assembly

Deprotection of α amino groups	
1. TFA/DCM (70/30) + Ethanedithiol 0.25%	1 min
2. TFA/DCM (70/30) + Ethanedithiol 0.25%	15 min
Neutralization steps	
3. Isopropanol	1 min
4. DCM	2 × 1 min
5. DMF	1 min
Coupling reaction	
6. Boc amino acid in 25% DMF/DCM	2 eq. 30 min. after 15 min
DIEA	6 eq., add NMP
BOP	2 eq.
7. DCM	3 × 1 min

of interest to test the activity of these peptides in other species in order to assess its generality.

REFERENCES

[1] Hietter, H., Van Dorssclaer, A., Green, B., Denoroy, L., Hoffmann, J. and Luu, B. (1990) Eur. J. Biochem. 187, 241–247.
[2] Lagueux, M., Lwoff, L., Meister, M., Goltzene, F. and Hoffmann, J. (1990) Eur. J. Biochem. 187, 249–254.
[3] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149.
[4] Plaue, S. and Briand, J.P. (1988) in: Synthetic Polypeptides as Antigens (Van Regenmortel, M.H.V., Briand, J.P., Muller, S. and Plaue, S. eds.) pp. 41–94, Elsevier, Amsterdam.
[5] Castro, B., Dormoy, J.R., Evin, G. and Selve, C. (1975) Tetrahedron Lett. 14, 1219–1222.
[6] Kaiser, E., Colescott, R.L. Bossinger, C.D. and Cook, P.I. (1970) Anal. Biochem. 34, 595–598.
[7] Usherwood, P.N.R., Giles, D. and Suter, C. (1980) in: Insect Neurobiology and Pesticide Action, Neurotox 79, pp. 115–128, Soc. Chem. Ind., London.
[8] Ichinose, I. and McAdoo, D.J. (1988) J. Neurosci. 8, 3891–3900.
[9] Loh, Y.P., Brownstein, M.J. and Gainer, H. (1984) Annu. Rev. Neurosci. 7, 189–222.
[10] Douglass, J., Civelli, O. and Herbert, E. (1984) Annu. Rev. Biochem. 53, 665–715.
[11] Land, H., Schutz, G., Schmale, H., and Richter, D. (1982) Nature 295, 299–305.
[12] Gubler, U., Monahan, J.J., Lomedico, P.T., Bhatt, R.S. and Collier, K.J., Hoffman, B.J., Bohlen, P., Esch, F., Ling, N., Zeytin, F., Braszeau, P., Poonian, M.S. and Gage, L.P. (1983) Proc. Natl. Acad. Sci. USA 80, 4311–4314.
[13] Nagle, G.T., de Jong-Brink, M., Painter, S.D., Bergamin-Sassen, M.M.J., Blankenship, J.E. and Kurosky, A. (1990) J. Biol. Chem. 36, 22329–22335.
[14] Schwartz, T.W. (1986) FEBS Lett. 200, 1–10.
[15] Colombaioni, L.D., Paupardin-Tritsch, D., Vidal, P.P. and Gerschenfeld, H.M. (1985) J. Neurosci. 5, 2533–2538.
[16] Steiner, D.F. and Chicago, M.D. (1978) Diabetes 27, 145–148.
[17] Dorn, A., Rinne, A., Bernstein, H.-G., Hahn, H.-J. and Ziegler, M. (1983) J. Hirnforsch. 24, 495–499.