Cyclosporin A selectively reduces the functional expression of Kir2.1 potassium channels in *Xenopus* oocytes

Haijun Chen[a], Yoshihiro Kubo[b], Toshinori Hoshi[c], Stefan H. Heinemann[a,*]

[a] *Max-Planck-Gesellschaft, Arbeitsgruppe Molekulare und zelluläre Biophysik an der Friedrich-Schiller-Universität Jena, Drackendorfer Strasse 1, D-07747 Jena, Germany*
[b] *Department of Neurophysiology, Tokyo Metropolitan Institute for Neuroscience, Musashidai 2-6, Fuchu-city, Tokyo 183, Japan*
[c] *Department of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242, USA*

Received 23 December 1997

Abstract The immunosuppressant cyclosporin A (CsA) reduced the functional expression of Kir2.1 potassium channels in *Xenopus* oocytes in a dose-dependent manner with an IC_{50} of 11 μM when the oocytes were incubated with CsA after RNA injection. FK506 was less effective than CsA; cyclosporin H, a non-immunosuppressive derivative of CsA, did not have a significant effect. CsA did not impair protein synthesis since other potassium channel types (Kir1.1, Kv1.1, Kv1.4) were much less sensitive to CsA. Our results suggest that the functional expression of Kir2.1 channels is facilitated by the peptidyl-prolyl isomerase cyclophilin. The observations illustrate a new role of CsA in regulation of membrane ion transport, and may provide an alternative explanation for CsA-induced side effects in clinical use.

Key words: *Xenopus* oocyte; Immunosuppression; Cyclosporin A; Cyclophilin; Peptidyl-prolyl isomerase; Potassium channel

1. Introduction

The primary amino acid structures of various ion channel subunits are now known, however, their transmembrane topologies have not been well defined. Neither is it known how these subunits are folded, assembled, and inserted into the cell membranes. As in other proteins, enzymes may participate as catalysts in the folding of channel-forming proteins [1]. For example, the *cis-trans* isomerization of prolyl peptide bonds is known to be a critical step in the folding of several proteins [2,3]. Thus, this process may also be important for the assembly of functional ion channel complexes. *Cis-trans* isomerization is a slow process, often acting as a rate-limiting step in protein folding, but it can be greatly accelerated by peptidyl-prolyl isomerases [4]. Two principal classes of the isomerases belonging to the cyclophilin (CyP) and FK506-binding protein (FKBP) families are now well characterized [5–8]. CyPs have been directly implicated in the folding of several proteins and peptides [5,9,10].

In addition to their roles in protein folding acting as peptidyl-prolyl isomerases, CyP and FKBP are also targets of two powerful immunosuppressant drugs, cyclosporin A (CsA) and FK506, respectively. CsA is clinically used to prevent graft rejection after organ transplantation. CsA and FK506 are thought to induce the immunosuppressant effects by forming CyP-CsA and FKBP-FK506 complexes, which in turn inhibit the Ca^{2+}-dependent phosphatase calcineurin [11]. While the calcineurin-mediated pathways of the CsA and FK506 actions are inhibited by the immunosuppressants at low concentrations (5–100 nM and 0.5–10 nM, respectively [11]), much higher concentrations are needed to inhibit the potency of CyP or FKBP to act as peptidyl-prolyl isomerases (1–10 μM [4]).

It was recently shown that CsA significantly reduces the surface expression in *Xenopus* oocytes of homo-oligomeric α7 neuronal nicotinic and type 3 serotonin ($5HT_3$) receptors, suggesting that the peptidyl-prolyl isomerase CyP is critically involved in the expression of these proteins [12,13]. CsA also inhibits apical secretory K^+ channels in rabbit cortical collecting tubule principal cells [14]. Many potassium channel proteins show proline-rich regions, which may be affected by the peptidyl-prolyl isomerases. The inward rectifier potassium channel Kir2.1 (IRK1), for example, contains 12 proline residues in the C-terminal domain and one proline in the core domain (M1, P-domain, and M2) [15]. We therefore hypothesized that peptidyl-prolyl isomerases may be involved in the functional expression of potassium channels. We examined whether Kir2.1 potassium channel expression is dependent on the activities of the peptidyl-prolyl isomerases by using CsA and FK506 as selective blockers of CyP and FKBP, respectively. We report here that CsA specifically reduces the functional expression of the Kir2.1 channels in *Xenopus* oocytes without influencing the electrophysiological properties of the expressed channels. The results indicate that neither a simple block of functional channels, a block of protein synthesis nor an inhibition of general posttranslational modification accounts for the effect of CsA to selectively inhibit Kir2.1 channel expression. The functional expression of Kir2.1 channels may involve CyP as peptidyl-prolyl isomerase or as molecular chaperone without involving a calcineurin-dependent pathway.

2. Materials and methods

2.1. Oocyte expression and solutions

Stage V oocytes were surgically obtained from *Xenopus laevis* anesthetized with 0.2% tricaine in ice water. Oocytes were injected with 50 nl (0.05–0.5 μg/μl) of RNAs encoding various potassium channel types as described [16]. Subsequently, oocytes were divided into control and experimental groups, each containing at least 10 cells. These groups of oocytes were incubated at 18°C in Barth's medium with or without various concentrations of immunosuppressant drugs. When the expression of Kir1.1 channels was assayed, Kir1.1 and control (Kir2.1) oocytes were incubated in modified Barth's medium with increased K^+ concentration (20 mM KCl and 69 mM NaCl instead of 1 mM KCl and 88 mM NaCl), which increased the survival rate of oocytes injected with Kir1.1 RNA. CsA was purchased from Sigma

*Corresponding author. Fax: (49) (3641) 304 542.
E-mail: ite@rz.uni-jena.de

PII S0014-5793(98)00028-3

(St. Louis, MO, USA), cyclosporin H (CsH) was a gift from Dr. G. Fischer (Max Planck Society, Halle, Germany), and FK506 was kindly provided by Fujisawa GmbH (Munich, Germany). From a stock solution of 4 mM (CsA in methanol:ethanol (1:1); CsH in methanol; FK506 in ethanol), immunosuppressants were added to the Barth's medium immediately after injection. Equivalent amounts of vehicle alone had no effects on channel expression. Whole-oocyte currents were recorded about 16 h after injection. Extracellular solutions for the recording of the Kir1.1 and Kir2.1 channel currents contained (in mM): 90 KCl, 3 $MgCl_2$, 10 HEPES (pH 7.2 with KOH). For other potassium channels, the bath solution contained: 107 NaCl, 10 KCl, 1.8 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH).

2.2. Generation of channel mutants

Point mutants in Kir2.1 channels were created by means of the Sculptor Kit (Amersham, Buckinghamshire, UK) using oligonucleotide DNA primers and single-stranded template DNA. The mutations were confirmed by sequencing the primer and the surrounding regions. To avoid unexpected mutations, two independent mutant clones were confirmed to have identical electrophysiological properties. Plasmid DNA was linearized and capped RNA was synthesized in vitro using commercially available kits (Ambion, Austin, TX, USA). RNAs encoding Kir2.1 mutants and the other channel types were generated with the following combinations of linearization enzyme and promoter: Kir2.1 and Kir2.1 mutants (*Xho*I, T3); Kir1.1 (GenBank accession number X72341) (*Mlu*I, SP6); rKv1.1 (accession number X12589) (*Pst*I, T7), rKv1.4 (accession number X16002) (*Sal*I, SP6).

2.3. Electrophysiological recording and data analysis

Recording of whole-oocyte currents was performed at room temperature (19–22°C) with a two-electrode voltage clamp amplifier (Turbo Tec 10CD, npi electronic, Tamm, Germany). Electrodes were filled with 2 M KCl and had resistances of 0.6–1.0 MΩ. Data were collected with the Pulse+PulseFit acquisition package (HEKA Elektronik, Lambrecht, Germany) running on an Apple Macintosh Quadra 650 computer. Statistical data are given as mean ± S.E.M. (N = number of oocytes; n = number of oocyte batches) unless stated otherwise.

In most cases, currents in oocytes expressing Kir2.1 or Kir1.1 channels were measured by using voltage ramps from +50 mV to −100 mV (300 ms) from a holding potential of 0 mV; the current value at −100 mV was taken as the measured signal, while the current at +50 mV was used for linear leakage correction for Kir2.1. For Kir1.1, leak correction was performed by replacing K^+ by Cs^+ in the bath solution; the current level at −100 mV obtained in Cs^+ solution was considered leakage current. For other potassium channels, the holding potential was −90 mV and a P/6 method with a leak holding potential of −90 mV and a leak scaling factor of −0.2 was used to subtract leak and capacitive currents. Peak current was determined at +50 mV. Due to the large scatter of the data, each experiment included a control group of oocytes from the same batch injected with Kir2.1 RNA in the absence and the presence of the corresponding concentration of immunosuppressant, i.e. the effect of CsA on various potassium channel types was measured with respect to Kir2.1, the effect of various drugs with respect to CsA.

The concentration-response curve of the CsA effect was described according to $f = (1+[CsA]^h/IC_{50}{}^h)^{-1}$ where h is the Hill coefficient, f is the fractional inhibition at various concentrations of CsA, [CsA], and IC_{50} provides a measure for the half-maximal inhibition concentration.

3. Results and discussion

3.1. CsA reduces the expression of functional Kir2.1 channels

To test the effect of CsA on the expression of Kir2.1 channels, two groups of oocytes were incubated in Barth's medium in the presence (experimental groups) and absence (control group) of various concentrations (0.1–50 μM) of CsA immediately after RNA injection. Channel expression was assayed by recording whole-oocyte currents using two-electrode voltage-clamp techniques. As shown in Fig. 1A, oocytes incubated in CsA showed smaller potassium currents through the Kir2.1 channels, but did not change the inward rectifying behavior, which reflects the channel blockage by Mg^{2+} and spermine [17,18]. Acute application of 20 μM CsA for about 1 h to oocytes expressing Kir2.1 channels failed to decrease the inward potassium currents (not shown), indicating that CsA did not block the functional channels but rather inhibited a process involved in channel formation.

CsA reduced the current amplitudes through the Kir2.1 channels in a dose-dependent manner. The data shown in Fig. 1B were obtained from 63 different batches of oocytes, each with about 30 cells. Each data point is a mean value of whole-oocyte currents at −100 mV normalized by the respective control values, representing the expression level of Kir2.1 channels in the presence of the indicated concentrations of CsA (n is indicated for each data point). A dose-response fit with a Hill coefficient of $h = 1$ yielded an IC_{50} estimate of 10.7 μM. This concentration dependence of the CsA action on Kir2.1 is similar to the effects of CsA on the expression of

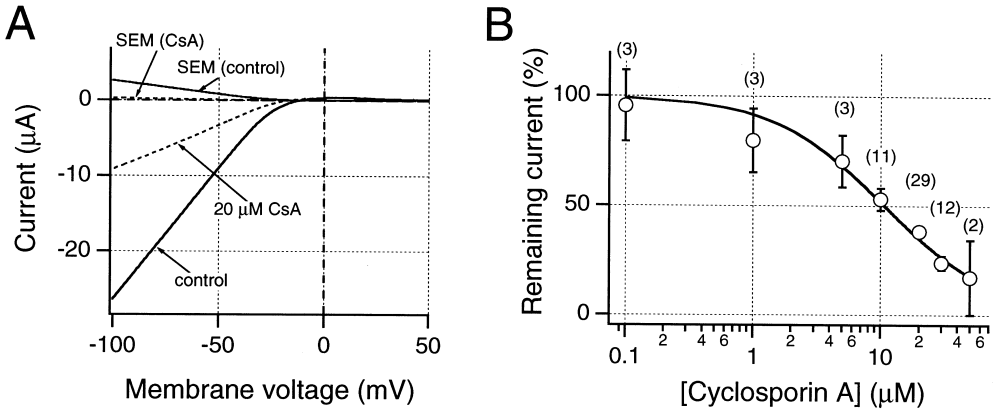

Fig. 1. CsA reduces the expression of functional Kir2.1 channels in *Xenopus* oocytes. A: Whole-oocyte currents in response to voltage ramps (300 ms) were measured under two-electrode voltage clamp approximately 16 h after RNA injection. Mean current traces from 21 control oocytes (solid curves) and mean current traces from 22 oocytes which were incubated in Barth's medium containing 20 μM CsA (dashed curves) immediately after RNA injection are compared. In addition, the S.E.M. values are shown indicating the variability of current within one batch of oocytes. Linear leak correction was performed assuming that the current at +50 mV was leakage current with a reversal potential of 0 mV. B: CsA reduces the Kir2.1 channel expression in a dose-dependent manner. The curve is the result of a dose-response fit with a Hill coefficient of $h = 1$, yielding an IC_{50} value of 10.7 μM. Each point represents the remaining current level in the presence of the indicated concentration of CsA. The number of oocyte batches used to determine the data points are indicated; for each batch at least 20 oocytes were used.

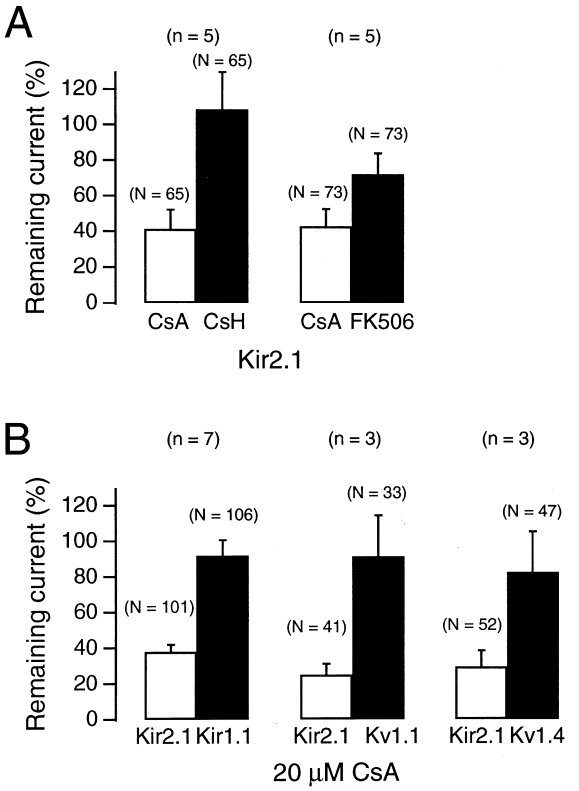

Fig. 2. A: Effects of CsH and FK506 on the expression of Kir2.1 channels. The effects of 20 μM CsH and FK506 on Kir2.1 channel expression are compared (in identical batches of oocytes) with the effect of 20 μM CsA (open bars). *n* indicates the number of batches and *N* the total number of oocytes. The error bars are estimates for the standard error of the mean. B: Effects of 20 μM CsA on the expression levels of Kir1.1, rKv1.1, and rKv1.4 channels, compared with the expression of Kir2.1.

α7 and $5HT_3$ receptors in oocytes [12]. The effect in this concentration range (μM range), based on previous studies of collagen triple-helix formation [19], the folding of transferrin [20], and T-cell signalling [21], suggests a direct inhibition of the activity of endogenous CyP involved in protein folding. The effect of CsA mediated by inhibition of calcineurin is reported to be effective at lower concentrations (<100 nM) [11]. The variability of the CsA effect among different batches of oocytes might be caused by the variability of endogenous [CyP] which has been estimated to be in the range of 10–30 μM [21].

3.2. The effects of CsH and FK506 on the expression of Kir2.1 channels

It is known that CsA inhibits the T-cell receptor signal transduction pathway via the formation of CsA-CyP complexes which inhibit calcineurin [11,22]. To investigate the possible role played by the known intracellular targets of CsA, CyP and calcineurin, the effect of a non-immunosuppressive derivative of CsA, cyclosporin H (CsH), on the expression of Kir2.1 channels was examined. Although similar to CsA in structure, CsH does not inhibit the prolyl isomerases and it does not complex with CyP to inhibit calcineurin [23]. Incubation of injected oocytes in 20 μM CsH for 16 h failed to cause a significant reduction of currents (Fig. 2A), indicating that CsH does not influence channel expression. This result supports the notion that CsA does not exert its effect in a non-specific manner.

Two classes of prolyl isomerases, CyP and FKBP, are involved in cellular *cis-trans* isomerization of prolyl peptide bonds [5–8]. To examine which class of prolyl isomerases is involved, we studied the effect of FK506 on Kir2.1 channel expression. FK506 is about 10 times more potent than CsA against its cognate isomerase FKBP [11], which, however, is not abundantly expressed in stage V–VI oocytes of *Xenopus laevis* [24]. Consistent with these expectations, 20 μM FK506 induced a smaller effect than CsA (Fig. 2A).

3.3. CsA does not block protein synthesis and processing

The CsA-induced reduction of channel expression might result from a non-specific blockade of protein synthesis in oocytes. According to this hypothesis, a non-specific effect of CsA on protein synthesis in oocytes should cause the blockade of expression of any RNA injected. Thus, we examined how CsA affected other potassium channel proteins and found that CsA (20 μM) failed to affect functional expression of the Kv1.1 and Kv1.4 channels (Fig. 2B). These channels are activated by membrane depolarization and generate outwardly rectifying potassium currents. Electrophysiological channel properties such as activation and inactivation kinetics and voltage dependence of activation of these channels were not altered (data now shown). Expression of the inward rectifier channel Kir1.1 which is expressed in kidney was not significantly affected by 20 μM CsA (Fig. 2B). At higher concentrations a small reduction of expression was observed (not shown). These results indicate that the process of protein translation leading to Kir2.1 channels is not suppressed via the inhibition of CyP activity by CsA. Similar results were obtained by Helekar et al. [12] showing that the expression

Table 1
Effects of CsA on Kir2.1 mutants

Mutant	[CsA] (μM)	Rel. current (%) (wild type)	*N*	Rel. current (%) (mutant)	*N*
Y242F	10	62 ± 12	17	59 ± 7	41
	20	21 ± 5	26	27 ± 5	23
Y366F	10	44 ± 6	35	57 ± 11	39
	10	62 ± 12	27	43 ± 11	23
S3A	30	37 ± 8	28	29 ± 7	31
T3A	30	37 ± 8	28	35 ± 8	30
S357A	30	37 ± 8	28	35 ± 6	20
T383A	30	37 ± 8	28	22 ± 5	29
S426N	20	54 ± 12	24	48 ± 8	26

The remaining current level ± S.D. is listed for the indicated concentrations of CsA and the Kir2.1 mutants. For comparison, the effect on wild type Kir2.1 channels, obtained in the same batch of oocytes, is included. *N* indicates the total number of oocytes used.

of hetero-oligomeric nicotinic receptors was not affected by CsA. Therefore, the CsA-mediated blockade of CyP activity is likely to specifically interfere with the process of Kir2.1 channel folding and insertion into the membrane or with other specific posttranslational mechanisms.

As proline residues are expected to be targets for peptidyl-prolyl isomerases and as they might constitute parts of interaction sites, we generated a double mutant in which two proline residues in a PxxP motif were replaced by alanine, Kir2.1:P351A·P354A. This mutation, however, did not eliminate the CsA effect (20 μM CsA reduced the expression to $54 \pm 7.5\%$ ($n = 5$, $N = 97$) while in the same batches of oocytes expression of wild type Kir2.1 was reduced to $57 \pm 7.9\%$ ($n = 5$, $N = 78$)). However, these experiments do not rule out the possibility that other proline residues play a role as parts of interaction sites. Interestingly, CsA has a significantly weaker effect on the expression of Kir1.1 channels which share a similar structure of two membrane-spanning segments with Kir2.1 (40% amino acid identity). In the C-terminal part of the Kir2.1 polypeptide there are four more proline residues compared with Kir1.1. Therefore, it is conceivable that the different distribution of prolines in the C-terminal polypeptide parts might result in the different CsA effects on channel expression; the potential role of proline residues for the CsA effect needs to be elucidated by further site-directed mutagenesis of Kir2.1.

It is possible that CsA regulates Kir2.1 indirectly by affecting a cellular regulatory protein which in turn affects the channel. Kir2.1 contains several putative phosphorylation sites and the phosphorylation states of the channel may be important in the functional expression. To determine whether the CsA effect was mediated by the activities of endogenous tyrosine kinases, protein kinase A (PKA) or protein kinase C (PKC), we generated several mutants eliminating the two putative tyrosine phosphorylation sites (Y242F, Y366F) or several PKA/PKC sites (S3A, T6A, S357A, T383A, S426N). None of these mutations changed the channel sensitivity to CsA significantly (see Table 1).

CsA is a cyclic peptide widely used in the prevention and treatment of organ transplant rejection, but with many severe side effects. Previous studies suggested that CsA may interfere with ion transport through cell membranes [25,26]. Therefore, the side effects of CsA may be mediated by ion channels, in particular by Kir2.1 as shown in this study. This side effect of CsA could involve multiple organs since potassium channels of the Kir2 subfamily are expressed in several human tissues (skeletal muscle, heart, brain, kidney [27]) and they play important roles in setting the resting membrane potential, buffering external K^+, and modulating action potential waveforms [28]. It is noteworthy that the apparent IC_{50} value of about 10 μM for the reduction of Kir2.1 expression by CsA is similar to the concentration of CsA clinically used in organ transplant recipients. Considering that the inward rectifier channels are implicated in numerous cellular functions, it is possible that the inhibition of Kir2.1 channel expression by CsA may underlie some of the side effects of CsA in clinical use.

Acknowledgements: We thank A. Roßner and S. Arendt for technical assistance and R. Schönherr for helpful comments. We thank the following colleagues for providing cDNA clones: J. Tytgat (rKv1.1), M. Koenen (rKv1.4), U. Brändle, and R. Schöpfer (Kir1.1). This project was supported in part by HFSP and SFB 197 'Biological and Model Membranes'.

References

[1] Gething, M.J. and Sambrook, J. (1992) Nature 355, 33–45.
[2] Brandts, J.F., Halvorson, H.R. and Brennan, M. (1975) Biochemistry 14, 4953–4963.
[3] Schmid, F.X. and Baldwin, R.L. (1978) Proc. Natl. Acad. Sci. USA 75, 4764–4768.
[4] Lang, K., Schmid, F.X. and Fischer, G. (1987) Nature 329, 268–270.
[5] Fischer, G., Wittmann-Liebold, B., Lang, K., Keifhaber, T. and Schmid, F.X. (1989) Nature 337, 476–478.
[6] Takahashi, N., Hayano, T. and Suzuki, M. (1989) Nature 337, 473–475.
[7] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) Nature 341, 758–760.
[8] Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. and Sigal, N.H. (1989) Nature 341, 755–757.
[9] Lang, K. and Schmid, F.X. (1988) Nature 331, 453–455.
[10] Fischer, G. and Schmid, F.X. (1990) Biochemistry 29, 2205–2213.
[11] Liu, J., Farmer Jr., J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[12] Helekar, S.A., Char, D., Neff, S. and Patrick, J. (1994) Neuron 12, 179–189.
[13] Helekar, S.A. and Patrick, J. (1997) Proc. Natl. Acad. Sci. USA 94, 5432–5437.
[14] Ling, B.N. and Eaton, D.C. (1993) Kidney Int. 44, 974–984.
[15] Kubo, Y., Baldwin, T.J., Jan, Y.N. and Jan, L.Y. (1993) Nature 362, 127–133.
[16] Stühmer, W., Terlau, H. and Heinemann, S.H. (1992) in: Practical Electrophysiological Methods (Kettenmann, H. and Grantyn, R., Eds.), pp. 121–125, Wiley-Liss, New York.
[17] Fakler, B., Brändle, U., Bond, C., Glowatzki, E., Koenig, C., Adelman, J.P., Zenner, H.-P. and Ruppersberg, J.P. (1994) FEBS Lett. 356, 199–203.
[18] Ficker, E., Taglialatela, M., Wible, B.A., Henley, C.M. and Brown, M. (1994) Science 266, 1068–1072.
[19] Steinmann, B., Bruckner, P. and Superti-Furga, A. (1991) J. Biol. Chem. 266, 1299–1303.
[20] Lodish, H.F. and Kong, N. (1991) J. Biol. Chem. 266, 14835–14838.
[21] Walsh, C.T., Zydowsky, L.D. and McKeon, F.D. (1992) J. Biol. Chem. 267, 13115–13118.
[22] Clipstone, N.A., Fiorentino, D.F. and Crabtree, G.R. (1994) J. Biol. Chem. 269, 26431–26437.
[23] Handschumacher, R.E., Harding, M.W., Rice, J., Gruggs, R.J. and Speicher, D.W. (1984) Science 226, 544–547.
[24] Kobrinsky, E., Ondrias, K. and Marks, A.R. (1995) Dev. Biol. 172, 531–540.
[25] Cappasso, G., Rosati, C., Ciani, F., Giordano, D.R., Russo, F. and Desanto, N.G. (1990) Am. J. Hypertens. 3, 204–210.
[26] Weir, M.R., Klassen, D.K., Shen, S.Y., Sullivan, D., Buddemeyer, E.U. and Handwerger, B.S. (1990) Transplantation 49, 41–47.
[27] Tang, W., Qin, C. and Yang, X. (1995) Rec. Chan. 3, 175–183.
[28] Doupnik, C.A., Davidson, N. and Lester, H.A. (1995) Curr. Opin. Neurobiol. 5, 268–277.