FEBS 22191 FEBS Letters 453 (1999) 351–355

Specific sequence-directed anti-bilitranslocase antibodies as a tool to detect potentially bilirubin-binding proteins in different tissues of the rat

Lucia Battiston, Annalisa Macagno, Sabina Passamonti, Fulvio Micali, Gian Luigi Sottocasa*

Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Via Giorgieri 1, I-34100 Trieste, Italy

Received 16 April 1999; received in revised form 17 May 1999

Abstract The hypothesis that the uneven distribution of bilirubin in the organism, which occurs in hyperbilirubinemia, could reflect an uneven distribution of bilirubin-binding proteins was tested by searching for peptides containing the bilirubin-binding motif identified in bilitranslocase (Battiston et al., 1998). In the rat, positive proteins bands were found to be present only in the liver, gastric mucosa and central nervous system. The electrophoretic mobilities of the positive compounds in the liver and stomach were identical to that of purified bilitranslocase (38 kDa). In the brain, on the contrary, two peptides were found with molecular masses of 79 and 34 kDa, respectively. Their distribution pattern in the central nervous system was different for each of them.

Key words: Bilitranslocase; Bilirubin; Organic anion transport; Liver transport; Tissue distribution of bilitranslocase

1. Introduction

Recently it was shown that the amino acid sequence EDSQGQHLSSF, present in the primary structure of bilitranslocase, corresponds to the central part of the bilirubin-binding site of the carrier [1,2]. The original assumption was based on the following observations: (a) the sequence in question is part of a motif conserved in ancient biliproteins such as phycocyanins whose prosthetic group is chemically related to bilirubin and (b) the motif, in phycocyanins, is located in proximity of the pigment [3]. Subsequently, it was possible to show directly that the immobilised peptide could efficiently compete with albumin for bilirubin-binding [4]. In addition, a sequence-specific antibody directed against the sequence mentioned above not only identified both purified bilitranslocase [1] and a single polypeptide with an identical mobility in solubilised plasmamembrane vesicles from rat liver but also inhibits the electrogenic sulfobromophthalein transport in this fraction, an activity specific for this translocator [5]. The kinetics of inhibition are depressed by bilirubin and nicotinate [1]. The calculated dissociation constants for the bilirubin- and nicotinate-bilitranslocase complexes were found to be 2 and 11 nM, respectively. These two values, in the nM range, are adequate for the function of bilitranslocase, which should be able to handle a substrate whose free concentration in plasma is maintained at an extremely low value due to the presence of serum albumin [6]. Such values, on the other hand, point to a very high affinity of the sequence in question for unconjugated bilirubin and suggest the possibility that the motif could be present also in other proteins potentially endowed with high affinity bilirubin-binding.

The availability of antibodies directed against the sequence of interest offered the opportunity to investigate the tissue distribution of bilitranslocase or, anyway, of proteins with an identical epitope and thus potentially capable of bilirubin-binding. This approach allows us to test the hypothesis that the tissue distribution pattern of proteins endowed with the ability to bind the pigment with high affinity (possibly contributing to its accumulation in certain regions of the organism) may account for the uneven distribution of the pigment, observed particularly during severe jaundice.

Data presented in this paper show that bilitranslocase (or at least a protein with identical mobility) is present not only in the liver but also in the gastric mucosa of the rat. In the central nervous system, two additional positive protein bands were found and, on the basis of their electrophoretical mobility, apparent molecular masses of 79 and 34 kDa was calculated. None of the two proteins was evenly distributed in the central nervous system (CNS) and, in addition, each component was found to be distributed according to its own pattern.

2. Materials and methods

2.1. Preparation of the biological material

The different tissues were all homogenised (10%) in 0.25 M sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA) using a motor-driven teflon glass Potter homogeniser. In the case of skeletal muscle, heart and uterus, sucrose solution was substituted with 0.15 M KCl buffered with 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA. At the end of the homogenisation, if necessary, neutralisation was carried out by adding drops of 1 M Tris base. The homogenates were centrifuged for 15 min at $5000\times g$ at 4°C. The supernatants were again spun down at $105\,000\times g$ for 1 h at the same temperature. The sediments were resuspended in an appropriate volume of homogenisation medium.

The microsomal fractions obtained as described usually collect at least 50% of the total plasmamembrane of the cells. In this way, we restricted our study to membranous material collected in these fractions disregarding nuclear and mitochondrial membranes as well as soluble proteins.

2.2. Multiple antigen peptide (MAP) generation and antibody production and purification

An undecapeptide (EDSQGQHLSSF) corresponding to sequence 65–75 of bilitranslocase [1] was synthesised coupled to an octalysine core to generate a MAP according to Tam [7]. The chemical synthesis was generously carried out for us by Prof. Renato Gennaro, University of Udine (Italy). Antibodies against the MAP were obtained in rabbits. The sequence-specific antibodies were purified by affinity

*Corresponding author. Fax: (39) (40) 6763691.
E-mail: sottoca@bbcm.univ.trieste.it

Abbreviations: CNS, central nervous system; MAP, multiple antigen peptide; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin

PII: S0014-5793(99)00736-X

chromatography using Affi-Gel 10 (Bio-Rad Laboratories, CA, USA)-coupled MAP (3.5 mg/ml). The elution from the column was carried out with an acid buffer (glycine-HCl, pH 2.5) and immediately neutralised with 1 M Tris pH 8. In order to minimise adsorption to test tubes, bovine serum albumin (BSA) (1.5 mg/ml) (Sigma, St. Louis, MO, USA) was added to the purified antibodies and, after enzyme-linked immuno adsorbent assay titration, stored at −20°C.

2.3. Electrophoretic and Western blotting analysis

The post-mitochondrial fractions obtained as described above, rat liver plasmamembrane vesicles [8] and purified bilitranslocase [9] were denatured in 2.5% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol, boiled for 5 min and separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. The proteins were electrophoretically transferred on an Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MO, USA) according to Gershoni and Palade [10]. As a primary antibody, affinity chromatography-purified anti-MAP antibodies, at the final concentration of 0.66 μg IgG/ml in the blocking buffer (Tropix, Bedford, MO, USA), were used. The incubation was performed overnight at 4°C, under mild agitation. The secondary antibody was 1:1000 diluted alkaline phosphatase-linked anti-rabbit IgG (Sigma, St. Louis, MO, USA). This second incubation lasted 1 h at room temperature. Extensive washing with phosphate-buffered saline (75 mM phosphate buffer, pH 7.4, 150 mM NaCl) containing 0.1% Tween 20 and 0.2% I-Block (Tropix, Bedford, MO, USA) was carried out. After a final washing in 10 mM diethanolamine buffer (pH 10) containing 1 mM $MgCl_2$, the antibody-binding was detected by the enhanced chemiluminescence technique using CSPD as substrate (Tropix, Bedford, MO, USA). The light intensity was determined by exposing X-ray films (Kodak, Rockester, NY, USA) to the membranes for 1–5 min.

2.4. Immunochemical microscopy

Tissue fixation was carried out with 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) overnight at 4°C. The specimens were washed in several changes of the same phosphate buffer and then dehydrated in a graded series of ethanol solutions before embedding in paraffin. 5 μm thin sections were used for light microscopy and processed further on microscopy glasses. The specimens were exposed for 1 h at room temperature to a Tris-buffered saline (TBS) solution (10 mM Tris-Cl, 120 mM NaCl, pH 7.4) containing 1% BSA (Sigma, St. Louis, MO, USA) and 20% normal goat serum and then incubated overnight at 4°C with affinity chromatography-purified sequence-specific antibodies (final concentration of 0.66 μg IgG/ml) in TBS containing 0.1% Tween 20 (Sigma, St. Louis, MO, USA) and 5% normal goat serum. The specimens were washed with TBS containing 1% BSA and incubated for 2 h at room temperature with colloidal gold (20 nm) conjugated goat anti-rabbit IgG (British BioCell International, Cardiff Wales, UK) diluted 1:100 in TBS containing 1% normal goat serum, 5% foetal calf serum, 1% BSA and 0.1% Tween 20. The specimens were washed in TBS+BSA followed by two washes in distilled water. Gold particles were enhanced by treatment with a silver enhancer kit (British BioCell International, Cardiff Wales, UK). The specimens were mounted as such after dehydration.

3. Results

It is well known that a classical microsomal fraction collects more than 50% of the total plasmamembrane. Such a fraction could therefore represent a useful material to investigate the distribution of a plasmamembrane protein in different tissues. It was necessary, first of all, to check that a liver microsomal preparation was adequate for the detection of bilitranslocase. Fig. 1 shows the results of an experiment in which erythrocyte ghosts (negative control) [5], plasmamembrane vesicles and microsomes from rat liver were subjected to electrophoresis and Western blotting in comparison with purified rat liver bilitranslocase. Clearly, the positivity to the sequence-specific antibody and the corresponding mobility (around 38 kDa apparent molecular mass) were the same in all the three positive samples. It was hence concluded that the sensitivity of the method was adequate to detect the presence of bilitranslocase in a microsomal fraction.

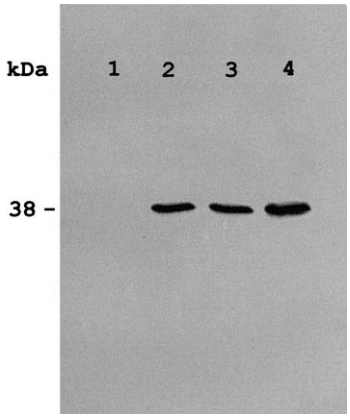

Fig. 1. Western blot of solubilised proteins from erythrocyte ghosts, plasmamembranes and microsomes from rat liver in comparison with purified hepatic bilitranslocase. Experimental conditions: in lane 1, 2 and 3, 60 μg of proteins from erythrocyte ghosts, plasmamembrane vesicles and microsomes was loaded. In lane 4, approximately 0.5 μg purified bilitranslocase was loaded. The electrophoresis and Western blot were performed as reported in Section 2.

On these grounds, using the same subcellular fraction, we extended the study to other tissues. Fig. 2 shows that gastric mucosa gives a positive protein band with a mobility identical to that of liver bilitranslocase. Conversely, the kidney and pancreas were devoid of any reactive polypeptide. In the central nervous tissue, two immuno-reactive polypeptides were detected. On the basis of their relative mobility, apparent molecular masses of 79 and 34 kDa were calculated. The possibility was considered that the two polypeptides found represented two different levels of aggregation of the same monomer. This was ruled out by the finding that the relative proportion of the two bands did not change when the same sample was pre-treated with 6 M urea (lane 6, added for comparison).

As for gastric mucosa, it should be mentioned that it was

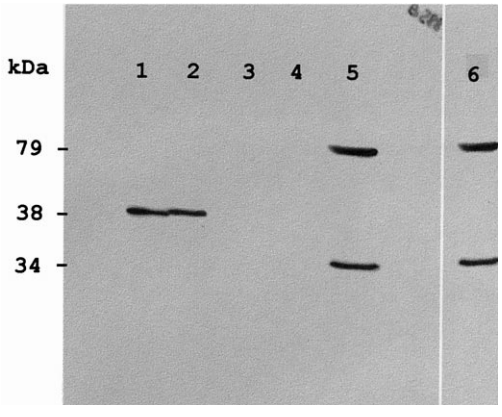

Fig. 2. Tissue distribution of the bilirubin-binding motif on solubilised proteins of the liver, gastric mucosa, pancreas, kidney cortex and whole brain post-mitochondrial fractions from rat. Experimental conditions: all wells were loaded with 60 μg of SDS-solubilised microsomal proteins. Lane 1: liver; lane 2: gastric mucosa; lane 3: pancreas; lane 4: kidney cortex, lane 5: whole brain; lane 6: whole brain pre-treated with 6 M urea. The electrophoresis and Western blot were performed as reported in Section 2.

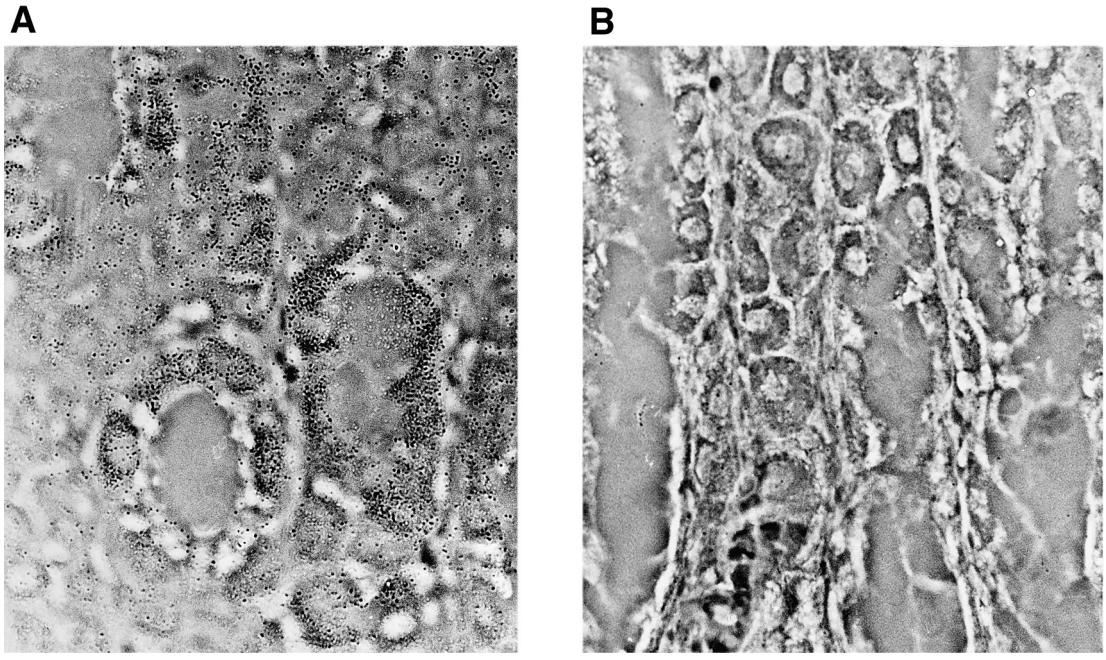

Fig. 3. Immuno-gold decorated microscopic sections of the rat gastric mucosa. Experimental conditions were as described under Section 2. (A) phase-contrast light microscopy of secreting gastric mucosal cells after immuno-gold decoration. (B) Control treated under the very same conditions with the only omission of the primary antibody. Final magnification ×1000.

obtained by mechanical scraping of the lumenal surface of the stomach and the resulting material is expected to be highly heterogeneous. A more precise assessment of the cellular type responsible for the data found could be obtained by immunochemistry. Such an approach showed that the protein targeted by the antibody is confined to epithelial cells (data not shown). At a high magnification, Fig. 3 shows a number of secretory cells stained with immuno-gold (A) and the appropriate control (B). Most cells gave a positive response although unstained or poorly stained elements were also present.

In view of the high level of structural complexity of the brain and in consideration of the fact that bilirubin tends to accumulate unevenly in this organ during severe jaundice [11,12], we thought it was interesting to study the distribution of the two proteins in different brain regions. To this aim, the experiment reported in Fig. 4 was carried out. All the wells were loaded with the same amount of proteins so that an approximately quantitative comparison can be done.

Starting from lane 2, where the two polypeptides are virtually equally abundant, one observes a progressive decrease of the high molecular mass compound and a mirror image pic-

Table 1
Tissue distribution of immuno-reactive peptides containing the bilirubin-binding motif in the rat

Tissue	38 kDa band	34 kDa band	79 kDa band
Liver	++	–	–
Kidney cortex	–	–	–
Oesophagus mucosa	–	–	–
Gastric mucosa	++	–	–
Duodenal mucosa	–	–	–
Jejunum mucosa	–	–	–
Colon mucosa			
-Ascending	–	–	–
-Transverse	–	–	–
-Descending	–	–	–
Spleen	–	–	–
Lungs	–	–	–
Pancreas	–	–	–
Skeletal muscle	–	–	–
Heart muscle	–	–	–
Myometrium	–	–	–
Erythrocytes	–	–	–
Whole brain	–	+	+
-Midbrain	–	++	+++
-Cerebral hemisphere	–	+	++
-Cerebellar hemisphere	–	+	+
-Brain stem	–	++	+
-Spinal cord	–	++	+

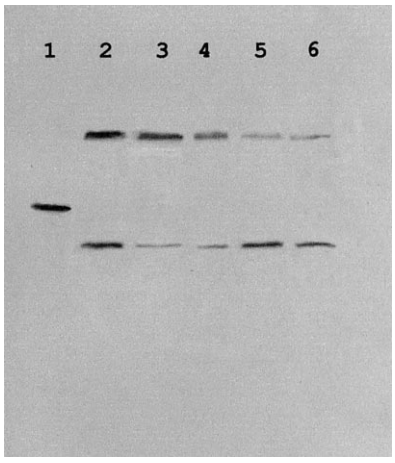

Fig. 4. Western blot carried out on solubilised proteins from a post-mitochondrial fraction of different rat brain regions and rat liver. Experimental conditions: 60 μg SDS-solubilised proteins per well. Lane 1: rat liver; lane 2: mid brain; lane 3: cerebral hemisphere; lane 4: cerebellar hemisphere; lane 5: pons and brain stem; lane 6: spinal cord. The electrophoresis and Western blot were performed as reported in Section 2.

ture is obtained for the low molecular mass one. It may therefore be concluded that the two immuno-reactive polypeptides with potential bilirubin-binding ability are unevenly distributed in the brain but according to different patterns.

Table 1 summarises the results obtained on a large number of tissues. Clearly, only liver and stomach showed a positive polypeptide with a mobility identical to that of isolated bilitranslocase. On the other hand, the other two immuno-responsive proteins found in the nervous system were absent in all the other tissues and organs analysed.

4. Discussion

The data presented in this paper show that hepatic bilitranslocase is the only detectable protein in liver membranes containing the epitope targeted, which was shown to be located in the central part of the bilirubin-binding motif of the carrier [1,2]. Obviously, the data do not rule out the possible contribution of other proteins in the transport mechanism of unconjugated bilirubin either from the blood into the cytoplasm of the hepatocytes or from the cytoplasm into the bile [13–19]. Furthermore, it should be remembered that, in this case, the material analysed consisted of a classical microsomal fraction and that the experimental design restricted the field to the membranes co-sedimented there. As for the positive protein band found in gastric mucosa, the question remains if we were indeed dealing with bilitranslocase or else with a different protein with very similar immunological and electrophoretic characteristics. The possibility that the carrier could operate also at the gastric level may be understood in physiological terms, as connected with the hypothetical uptake of nicotinic acid in the stomach. Such an event could ensure an efficient uptake of this vitamin in the proximal portion of the digestive tract, thus avoiding bacterial utilisation in the lower part of it. This possibility, though hypothetical, is feasible in view of the very high affinity of bilitranslocase for nicotinate ($K_d = 11$ nM) [1].

A point which deserves discussion is the total absence of bilitranslocase or, anyway, other peptides with an identical immuno-reactivity in the kidney cortex. We reported in the past [20] that, in this tissue, bilitranslocase was present and localised in the basolateral plasmamembrane of proximal tubules. The kinetic characteristics of the carrier described in that paper were however different from those described for hepatic bilitranslocase in that the apparent K_m measured was 3–4 times higher in the kidney. One of the explanations suggested for this finding at that time was that renal bilitranslocase, although largely homologous to the hepatic protein, is partially different in its primary structure. The absence of reactivity with the sequence-specific antibodies seems to substantiate this view.

The finding that brain contains two different proteins giving a positive response to the treatment with the sequence-specific antibody opens the possibility that in the CNS, two additional protein components, with a potential bilirubin-binding ability, are present. For the time being, the identity of the two components, as well as their functions, are completely unknown. The antibody used allows to predict that cross-reacting proteins should contain at least part of the amino acid sequence present in the MAP used to raise the antibodies. Data banks searching for this characteristics (PIR 41, SBASE 2.0 and Swiss-Prot 29) revealed that the receptor for mineral corticoid expressed in the hippocampus of the rat [21,22] contains the sequence QHLSSF. The molecular mass of this protein, however, is reported to be 106.7 kDa. Always, in the rat, a homology of six consecutive residues (EDSQGQ) is reported for chromogranin B, expressed in the hypophysis, whose molecular mass is reported to be 77.9 kDa [23].

Looking for functional homologies, oatp, another organic anion transporting polypeptide, present in liver and kidney [15], has recently been reported to be present also in the CNS [24]. The absence, however, of the epitope targeted in the known sequence of the oatp family [25] would exclude the possibility for these proteins to be detected by our sequence-directed antibody.

Immunochemistry using specific antibodies separately directed to the two proteins will provide an insight to the tissue- and cellular localisation of them and, perhaps, offer suggestions to their function.

In perspective, these findings may acquire relevance in the human pathology considering that the two polypeptides identified here are potentially endowed with a bilirubin-binding capacity. It is tempting to speculate that the uneven distribution of unconjugated bilirubin observed in severe hyperbilirubinemia [11,12] may be related to the uneven distribution of the two proteins.

Acknowledgements: The AA wish to express their gratitude to Prof. Renato Gennaro for the preparation of MAP. The skilful technical assistance of Mr. G.C. Lunazzi and Dr. M. Stebel is also gratefully acknowledged. This research has been supported by grants of the Italian Ministry of University and Scientific and Technological Research (PRIN: Bioenergetica e Trasporto di Membrana). L. Battiston was a recipient of a post-doc fellowship from the University of Trieste.

References

[1] Battiston, L., Passamonti, S., Macagno, A. and Sottocasa, G.L. (1998) Biochem. Biophys. Res. Commun. 247, 687–692.
[2] Passamonti, S., Battiston, L. and Sottocasa, G.L. (1999) Mol. Membr. Biol. (in press).

[3] Schirmer, T., Bode, W. and Huber, R. (1987) J. Mol. Biol. 196, 677–695.
[4] Battiston, L., Macagno, A., Passamonti, S. and Sottocasa, G.L. (1997) Ital. Biochem. Soc. Trans. 9, 104.
[5] Baldini, G., Passamonti, S., Lunazzi, G.C., Tiribelli, C. and Sottocasa, G.L. (1986) Biochim. Biophys. Acta 856, 1–10.
[6] Pascolo, L., Del Vecchio, S., Koehler, R.K., Bayon, J.E., Webster, C.C., Mukerjee, P., Ostrow, J.D. and Tiribelli, C. (1996) Biochem. J. 316, 999–1004.
[7] Tam, J.P. (1989) Methods Enzymol. 168, 7–15.
[8] Van Amersvoort, Y.M.M., Sips, H.J. and Van Dam, K. (1978) Biochem. J. 174, 1083–1086.
[9] Sottocasa, G.L., Lunazzi, G.C. and Tiribelli, C. (1989) Methods Enzymol. 174, 50–57.
[10] Gershoni, J.M. and Palade, G.E. (1982) Anal. Biochem. 124, 396–405.
[11] Schenker, S., Hojumpa, A.M. and McCandless, D.W. (1986) in: Bile Pigments and Jaundice(Ostrow, J.D., Ed.), pp. 395–420, Marcel Dekker, New York and Basel.
[12] Lee, K. and Gartner, L.M. (1986) in: Bile Pigments and Jaundice (Ostrow, J.D., Ed.), pp. 373–394, Marcel Dekker, New York and Basel.
[13] Stremmel, W., Gerber, M.A., Glezerov, V., Thung, S.N., Kochwa, S. and Berk, P.D. (1983) J. Clin. Invest. 71, 1796–1805.
[14] Wolkoff, A.W., Sosiak, A., Greenblatt, H.C., Van Renswoude, J. and Stockert, R.J. (1985) J. Clin. Invest. 76, 454–459.
[15] Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) Proc. Natl. Acad. Sci. USA 91, 133–137.
[16] Goeser, T. et al. (1990) J. Clin. Invest. 86, 220–227.
[17] Kullak-Ublick, G.A., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) Hepatology 20, 411–416.
[18] Kanai, N., Lu, R., Bao, Y., Wolkoff, A.W. and Schuster, V.L. (1996) Am. J. Physiol. 270, F319–F325.
[19] Pascolo, L., Bayon, E.J., Cupelli, F., Ostrow, J.D. and Tiribelli, C. (1998) Biochem. J. 331, 99–103.
[20] Elias, M.M., Lunazzi, G.C., Passamonti, S., Gazzin, B., Miccio, M., Stanta, G., Sottocasa, G.L. and Tiribelli, C. (1990) Am. J. Physiol. 259, F559–F564.
[21] Kwak, S.P., Patel, P.D., Thompson, R.C., Akil, H. and Watson, S.J. (1993) Endocrinology 133, 2344–2350.
[22] Patel, P.D., Sherman, T.G., Goldman, D.J. and Watson, S.J. (1989) Mol. Endocrinol. 3, 1877–1885.
[23] Pohl, T.M., Phillips, E., Song, K.Y., Gerdes, H.H., Huttner, W.B. and Ruther, U. (1990) FEBS Lett. 262, 219–224.
[24] Angeletti, R.H., Bergwerk, A.J., Novikoff, P.M. and Wolkoff, A.W. (1998) Am. J. Physiol., C882–C887.
[25] Noe, B., Hagenbuch, B., Stieger, B. and Meier, P.J. (1997) Proc. Natl. Acad. Sci. USA 94, 10346–10350.