Volume 240, number 1,2, 65–70 FEB 06480 November 1988

The β-bungarotoxin-binding protein from chick brain: binding sites for different neuronal K^+ channel ligands co-fractionate upon partial purification

Ralf R. Schmidt and Heinrich Betz

ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

Received 29 August 1988; revised version received 23 September 1988

β-Bungarotoxin (β-Butx) is a presynaptically active neurotoxin which blocks neuronal A-type K^+ channels. Here, the efficient solubilisation and about 300-fold purification of the β-Butx-binding protein from chick brain were achieved by detergent extraction at high ionic strength followed by chromatography on DEAE Affigel Blue, β-Butx Affigel 102 and wheat germ agglutinin Sepharose. Binding of ^{125}I-labelled β-Butx to the purified protein was inhibited by two other K^+ channel ligands, dendrotoxin I and mast cell-degranulating peptide. It is concluded that the β-Butx-binding protein is a member of a family of voltage-gated K^+ channels which exhibit varying affinities for different polypeptide ligands.

β-Bungarotoxin; Dendrotoxin; Mast cell-degranulating peptide; K^+ channel; (Chick brain)

1. INTRODUCTION

K^+ channels are found in many animal tissues and represent a highly diverse family of membrane proteins. In excitable cells, voltage-dependent A-type K^+ channels control the duration of action potentials, cardiac pace-making and neurotransmitter release from presynaptic nerve terminals [1]. Due to the availability of channel mutants [2] and specific neurotoxins [3], A-type K^+ channels have recently become the object of intense biochemical and molecular genetic analysis. In *Drosophila*, functionally different A-type channels have been shown to result from alternative splicing of the highly complex '*shaker*' gene locus [4,5]. For the mammalian brain, evidence for heterogeneity of A-type channels mainly comes from electrophysiological and pharmacological studies [3].

β-Bungarotoxin (β-Butx), a basic protein from the venom of the snake *Bungarus multicinctus*, potently inhibits neurotransmitter release at the neuromuscular junction [6]. In the central nervous system, β-Butx is cytotoxic for cholinergic and GABA-(γ-aminobutyric acid)ergic neurons [7,8]. Recent electrophysiological data indicate that the mechanism of action of β-Butx involves binding to presynaptic A-type K^+ channels [9,10]. In chick brain membranes, ^{125}I-labelled β-Butx binds specifically to a large (430 kDa) membrane protein [11,12] composed of smaller subunits [13]. In support of its presumed K^+ channel nature, the β-Butx-binding protein exhibits a marked K^+ dependence for conformational stability upon solubilisation [12]. Furthermore, two other K^+ channel ligands, the facilitatory neurotoxins dendrotoxin I (Dtx-I, also called toxin I) from snake venom [14,15] and mast cell-degranulating (MCD) peptide from bee venom [16,17], inhibit binding of ^{125}I-β-Butx to synaptic membrane fractions in a non-competitive fashion [18]. Here we report the improved solubilisation and partial purification of

Correspondence address: H. Betz, ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

Abbreviations: β-Butx, β-bungarotoxin; Dtx-I, dendrotoxin I; MCD-peptide, mast cell-degranulating peptide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; WGA, wheat germ agglutinin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

the β-Butx-binding protein from chick brain and show that its interactions with Dtx-I and MCD-peptide are conserved during the isolation procedure.

2. MATERIALS AND METHODS

2.1. *Materials*

β-Butx was obtained from Miami Serpentarium (Miami, FL); Dtx-I and MCD-peptide were kindly provided by Drs M. Hollecker (Heidelberg) and M. Lazdunski (Nice).

2.2. *Preparation of membrane fractions*

Crude synaptic membranes were prepared from brains of newly hatched chicken by differential centrifugation as described [11] with minor modifications [19]. The final membrane pellet was resuspended in buffer A [20 mM K-Hepes (pH 7.4), 60 mM KCl, 2 mM Na-EDTA, 0.01% (w/v) NaN_3], frozen in liquid nitrogen and stored at −70°C.

2.3. *Preparation of detergent extract*

Thawed membrane fractions were adjusted to 0.45 M KCl, 1.5% (w/v) Triton X-100 and 25% (w/v) glycerol in buffer A and diluted 5-fold to a protein concentration of ≈2 mg/ml. After addition of protease inhibitors [19] and 2 h of slight stirring at 4°C, the suspension was diluted with 2 vols KCl-free buffer B [buffer A containing 0.8% (w/v) Triton X-100 and 0.2% (w/v) soybean phospholipid] and centrifuged at 48000 × *g* for 1 h. The resulting detergent extract was used freshly or stored frozen at −70°C.

2.4. *^{125}I-β-Butx-binding assays*

β-Butx was labelled with ^{125}I to an initial specific activity of 1200–1800 Ci/mmol [11]. Binding to membrane fractions was performed as described [18]. Buffer A contained 60 mM KCl instead of 115 mM; cellulose-acetate filters were replaced by GF/C glass-fiber filters presoaked in 0.3% (w/v) polyethylenimine in 0.135 mM Tris-Cl, pH 8.5. ^{125}I-β-Butx binding to soluble binding sites was determined using a modification [19] of the filter assay described by Bruns et al. [20]. Unless indicated otherwise, 1 mM $CaCl_2$ and 5 mM $MnCl_2$ were present in the assay. All binding data are corrected for nonspecific binding determined in the presence of 240 nM unlabelled β-Butx and represent the mean ± SE (indicated if larger than size of the symbol) of triplicate determinations. To remove leakage toxin, samples eluted from β-Butx affinity columns were incubated with CM-Sephadex C 25 (Pharmacia) prior to assay (batch incubation at 5°C for 30–50 h).

2.5. *Preparation of β-Butx affinity columns*

1–2 mg β-Butx per ml of gel were coupled to Affigel 15 (Biorad) according to the manufacturer's recommendations. Coupling was performed in 50 mM Na-Hepes (pH 7.4), 0.3 M KCl, 10 mM $CaCl_2$ for 1 h at 23°C with 70–90% efficiency.

For coupling of β-Butx to Affigel 102 (Biorad), the toxin was activated with the water-soluble carbodiimide, EDAC (Biorad) [19]. Coupling efficiencies determined by the addition of trace amounts of ^{125}I-β-Butx were 5–15%.

2.6. *Purification of the β-Butx-binding protein*

Purification of the β-Butx-binding protein was performed at 4°C. 9 ml DEAE Affigel Blue (Biorad) were equilibrated with buffer B in a column of 1 × 10 cm. Then 30–100 ml of detergent extract, diluted 2-fold to 75 mM KCl, were loaded onto the column at a flow rate of 14 ml/h. The column was washed with buffer B containing 100 mM KCl and eluted with the same buffer containing 200 mM KCl. (Elution by a gradient from 100 to 300 mM KCl released the binding activity in a single peak around 180 mM KCl.)

The eluate from the DEAE Affigel Blue column was dialysed against buffer C (buffer B containing 1 mM $CaCl_2$ and 5 mM $MnCl_2$ instead of EDTA) and loaded onto a β-Butx Affigel 102 column (1 ml) by cyclic overnight application at a flow rate of 14 ml/h. The column was washed sequentially with buffer C and with buffer C containing 100 mM KCl, and then eluted at a flow rate of 1 ml/h with buffer B containing 0.3 M KCl.

The eluate of the β-Butx affinity column was directly adsorbed onto a WGA column (1 ml) equilibrated with buffer B by cyclic overnight application (flow rate 10 ml/h). The column was washed with buffer B and eluted at 0.5 ml/h with buffer B containing 25 mM *N*-acetylglucosamine.

2.7. *Other methods*

Protein was determined by a modification [19] of the fluorescamine assay [21] using bovine serum albumin as a standard. Samples containing low protein concentrations were concentrated by precipitation according to Wessel and Flügge [22] and resolubilised in 5% (w/v) SDS.

3. RESULTS AND DISCUSSION

3.1. *Solubilisation of the β-Butx-binding protein*

Previous experiments have shown that the presence of K^+ is required for solubilisation of native ^{125}I-β-Butx-binding sites [12]. Here, the efficiency of solubilisation was increased from about 35 to >90% by using high ionic strength conditions (0.45 M KCl). Furthermore, addition of glycerol to the detergent extract markedly stabilized soluble ^{125}I-β-Butx-binding sites (half-life about 2 weeks at 5°C, not shown). Under these conditions the loss of binding activity upon freezing at −70°C was negligible.

Scatchard analysis showed that the affinity of ^{125}I-β-Butx binding to detergent extract was 2-fold lower than that to membranes (K_d about 500 pM for detergent extract, and 250 pM for membranes; see also fig.2).

3.2. *^{125}I-β-Butx binding to the solubilised binding protein: effect of ionic strength and divalent cations*

Previous studies of ^{125}I-β-Butx binding to detergent extract employed a tedious gel-filtration

assay procedure [12]. Here, this method was replaced by polyethylenimine filter binding which has been reported to recover nearly quantitatively other receptor sites [20]. The new assay not only gave higher specific binding values and a low unspecific filter binding signal, but also proved to be highly reproducible (SE of triplicate determinations 1–4%).

^{125}I-β-Butx binding to both detergent extract and membranes was strongly affected by the ionic strength (I) of the assay buffer (fig.1). Under the binding conditions used earlier [11,12], i.e. in the presence of 1 mM Ca^{2+}, a reduction of the KCl concentration from 115 mM (I = 0.142) to 39 mM (I = 0.066) increased specific binding to detergent extract by 4–5-fold. Scatchard analysis showed that this increase was mainly due to an increased apparent affinity (lower K_d) of the toxin rather than to a major alteration in the maximal number (B_{max}) of binding sites (fig.1, inset). Similar findings have also been made for other polypeptide ligands [23].

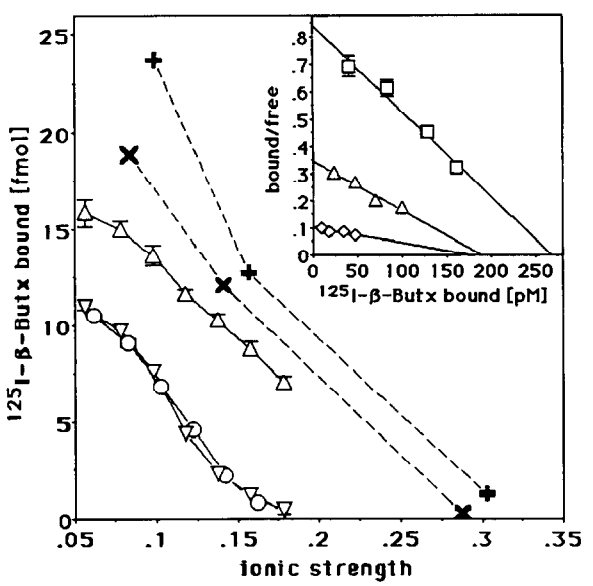

Fig.1. Effect of different ionic strength conditions and Mn^{2+} on ^{125}I-β-Butx binding. Binding to detergent extract was determined at different KCl concentrations (14–135 mM) in the presence of 1 mM $CaCl_2$ without (○) or with 5 mM $MgCl_2$ (▽), or with 5 mM $MnCl_2$ (△). For comparison, membrane binding (dashed lines) was tested at 60, 115 and 260 mM KCl in the presence of 1 mM $CaCl_2$ without (×) or with 5 mM $MgCl_2$ added (+). Inset: Scatchard plot of ^{125}I-β-Butx binding to detergent extract. Assay conditions were: 115 mM KCl, 1 mM $CaCl_2$ (◇); same but plus 2.5 mM $MnCl_2$ (△); 39 mM KCl, 1 mM $CaCl_2$ (□).

The inhibitory effect of high salt concentrations on ^{125}I-β-Butx binding was partially overcome by the addition of the divalent cation, Mn^{2+} (fig.1). This cation increased toxin binding to both detergent extract and membranes over the whole ionic strength range tested; the effect was, however, much more pronounced with the solubilised protein. Analysis of binding data revealed the increase of toxin binding to detergent extract by Mn^{2+} to result from a 2-fold decrease in apparent K_d (fig.1, inset).

Another divalent cation, Ca^{2+}, is known to bind to β-Butx with a K_d of 0.1–0.2 mM, thus altering the toxin's conformation [6] and its affinity for neuronal membranes [18]. The effect of Mn^{2+}, however, appears not to be mediated via this divalent cation-binding site of β-Butx, but rather by interaction with its membrane receptor. First, Mn^{2+} affected toxin binding to membranes much less than to the soluble protein (see fig.1). Second, Mn^{2+} could not be replaced by other divalent cations, i.e. Mg^{2+} or Sr^{2+}, the latter of which is known to substitute for the Ca^{2+} requirements of the snake toxin [6,11,24].

From these data, standard conditions for the determination of ^{125}I-β-Butx binding were established. As low ionic strength buffers increased unspecific filter binding of the labelled toxin, 60 mM KCl, 1 mM Ca^{2+} and 5 mM Mn^{2+} (I = 0.102) were routinely included in the binding assay.

3.3. *Purification of the β-Butx-binding protein*

In initial experiments, two types of β-Butx affinity resins were tested for retention of soluble β-Butx-binding sites. First, β-Butx was coupled to Affigel 15 via amino groups. The resulting resin contained about 15 nmol toxin per ml; its binding capacity was, however, only about 1.5 pmol/ml of toxin-binding sites. Derivatisation of Affigel 102 with EDAC-activated β-Butx produced gels of slightly better capacity, although coupling efficiencies were 5–10-times lower. A gel substituted with 6 nmol/ml of toxin retained 6–9 pmol of binding sites/ml.

From both types of gels, bound β-Butx-binding sites were efficiently eluted by 0.2–0.35 M KCl, but not by withdrawal of divalent cations with

Table 1

Purification of the β-Butx-binding protein

Purification step	Total protein (mg)	^{125}I-β-Butx bound[a] (fmol)	Specific activity (fmol/mg protein)	Yield (%)	Purification (-fold)
Detergent extract	52.5	2090	40	100	1
DEAE Affigel Blue eluate	8.0	1370	212	66	5.3
β-Butx Affigel 102 eluate	0.24	478	1990	23	50
WGA column eluate	0.075	400	5330	19	134

[a] Binding was determined under nonsaturating conditions (200 pM ^{125}I-β-Butx). Binding values are thus lower than those calculated from Scatchard plots

EDTA. Usually, a 20–40-fold enrichment in ^{125}I-β-Butx-binding sites was obtained (not shown). As β-Butx Affigel 102 exhibited a higher capacity and yield of binding sites, this resin was chosen for enriching larger amounts of the β-Butx-binding protein.

As the first step in a scaled-up purification procedure, detergent extract was concentrated on a DEAE Affigel Blue column which retained >80% of the binding sites present in the extract. About 80% of these sites were recovered upon salt elution with a 5-fold enrichment in specific ^{125}I-β-Butx-binding activity. Subsequent chromatography on a β-Butx Affigel 102 column resulted in a further 10-fold purification. After lectin affinity chromatography on WGA Sepharose, an overall 100–150-fold purification of the β-Butx-binding protein was achieved (table 1).

Purification did not significantly affect the ^{125}I-β-Butx-binding affinity of the toxin-binding protein (fig.2). For three different preparations, Scatchard analysis gave mean K_d values of 495 ± 165 and 415 ± 200 pM, and B_{max} values of 0.17 ± 0.05 and 14.3 ± 2.3 pmol/mg for detergent extracts and WGA column eluates, respectively.

From these data, and considering the about 3-fold enrichment of ^{125}I-β-Butx-binding sites during membrane preparation, the β-Butx-binding protein was purified about 300-fold over the tissue homogenate. This value is much lower than expected for a pure K^+-channel protein preparation. Assuming a molecular mass of 430 kDa [12], a theoretical toxin-binding activity of 2.3 nmol/mg protein is expected. Although inactivation of binding sites during lectin affinity chromatography and non-quantitative filter binding assays may mask higher purification factors (for discussions see [25]), further purification procedures certainly are required for establishing the polypeptide composition of the β-Butx-binding protein.

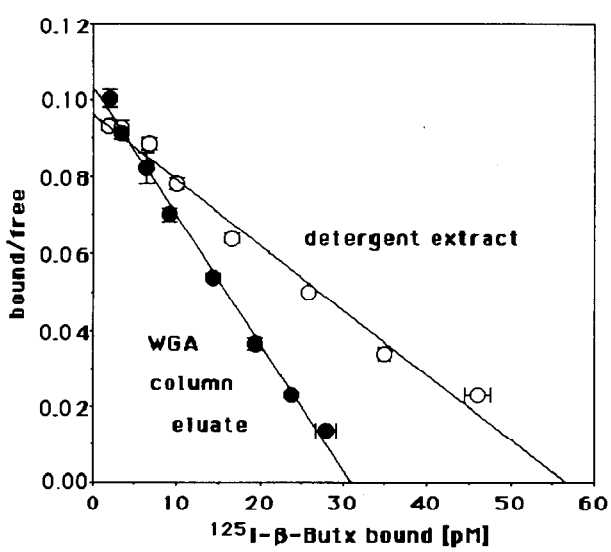

Fig.2. Scatchard analysis of ^{125}I-β-Butx binding (concentration range 0.02–2.5 nM) to detergent extract and WGA column eluate. In this experiment, K_d values determined with detergent extract and WGA column eluate were 580 and 300 pM, B_{max} values 0.24 and 14 pmol/mg protein, and Hill coefficients (n) 1.01 and 0.99, respectively.

3.4. *Pharmacology of the solubilised and partially purified β-Butx-binding protein*

Detergent extracts and WGA column eluates were tested for interaction with two other putative K^+ channel ligands, Dtx-I and MCD-peptide. Fig.3 shows the inhibition profiles of ^{125}I-β-Butx binding to a WGA column eluate by unlabelled β-Butx, Dtx-I and MCD-peptide. All toxins efficiently antagonized binding of the radiolabelled ligand. K_i values are summarized in table 2 and compared to those obtained with membrane fractions. It is evident that the affinity of all three toxins is unaltered or even increased upon solubilisation and purification. This is particularly pronounced in the case of MCD-peptide, where K_i values of 180 nM [18] and 60 nM (assay conditions of this study, see table 2) are observed with crude synaptic membranes, and 1–5 nM after solubilisation and partial purification, respectively.

The observation that Dtx-I and MCD-peptide binding sites copurify with the β-Butx-binding protein strongly supports the notion [18] that binding of these toxins indeed occurs at allosteric sites of the same membrane protein rather than at different A-type K^+ channel species. On the other hand, both binding studies [26] and chromatographic separation procedures [27] have provided clear evidence for the existence of physically separate high-affinity binding sites for ^{125}I-labelled Dtx-I in rat which exhibit high and low affinities for β-Butx. Furthermore, affinity purification of a Dtx-I and MCD-peptide binding protein from rat brain has been reported recently [28]. Interestingly, ^{125}I-Dtx-I binding to this putative K^+ channel is inhibited by β-Butx with an affinity (K_i 10 nM as calculated from [27]) at least 10-fold lower than that determined by direct radioligand binding to chick and rat brain membranes [18]. Thus, the β-Butx-binding site investigated here probably represents only one member of a family of toxin-sensitive A-type K^+ channels which differ in their relative affinities for β-Butx, Dtx-I and MCD-peptide. This interpretation is consistent with electrophysiological data on sensory and sympathetic neurons which indicate striking differences in the neurotoxin sensitivity of their A-type K^+ currents [17]. Moreover, a recent Northern blot analysis has also demonstrated considerable heterogeneity in putative A-type K^+ channel mRNAs in rat brain [29].

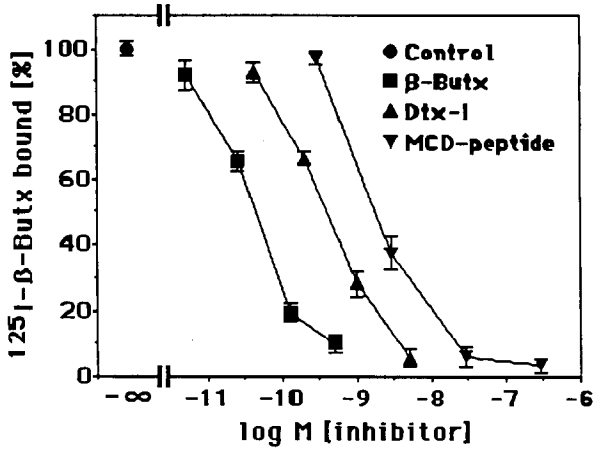

Fig.3. Inhibition of ^{125}I-β-Butx binding to WGA column eluate by β-Butx, Dtx-I and MCD-peptide. The inhibitors were diluted in buffer A containing 0.2% (w/v) bovine serum albumin and 0.01% (w/v) Triton X-100 and mixed with ^{125}I-β-Butx prior to addition of the WGA column eluate. Control binding was 4.85 ± 0.10 pmol/mg protein. From the concentrations causing half-maximal inhibition (IC_{50}) the apparent K_i values were calculated according to $K_i = IC_{50}/(L/K_d + 1)$ [30].

Table 2

Inhibition of ^{125}I-β-Butx binding by presynaptic neurotoxins

Toxin	K_i (nM)		
	Membranes	Detergent extract	WGA column eluate
β-Butx	0.05 ± 0.01	0.088 ± 0.024	0.046 ± 0.020
Dtx-I	0.33 ± 0.07	0.76 ± 0.18	0.31 ± 0.07
MCD-peptide	57 ± 20	4.6 ± 0.5	1.6 ± 0.4

Values represent means ± SE of three independent purifications except in the case of membranes where only a single experiment was performed

Acknowledgements: We thank Dr H. Rehm for collaboration in the initial stages of this work, Drs M. Hollecker and M. Lazdunski for the generous gifts of Dtx-I and MCD-peptide, Dr D. Göhmann for help in establishing the binding assay, Dr B. Schmitt and P. Schloß for discussions and Drs C.M. Becker and E.D. Gundelfinger for critical reading of the manuscript. R.R.S. is a fellow of the Landesgraduiertenförderungsprogramm Baden-Württemberg. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 317) and the Fonds der Chemischen Industrie.

REFERENCES

[1] Hille, B. (1984) in: Ionic Channels of Excitable Membranes, pp.99–116, Sinauer, Sunderland, MA.
[2] Salkoff, L. and Wymann, R. (1981) Nature 293, 228–233.
[3] Dolly, J.O. (1988) Trends Neurosci. 11, 186–188.
[4] Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Nature 331, 137–145.
[5] Pongs, O., Kecskemethy, N., Müller, R., Krah-Jentgens, I., Baumann, A., Kiltz, H.H., Canal, I., Llamazares, S. and Ferrus, A. (1988) EMBO J. 7, 1087–1096.
[6] Abe, T., Alema, S. and Miledi, R. (1977) Eur. J. Biochem. 80, 1–12.
[7] Rehm, H., Schäfer, T. and Betz, H. (1982) Brain Res. 250, 309–319.
[8] Halliwell, J.V. and Dolly, J.O. (1982) Neurosci. Lett. 30, 321–327.
[9] Petersen, M., Penner, R., Pierau, F.-K. and Dreyer, F. (1986) Neurosci. Lett. 68, 141–145.
[10] Dreyer, F. and Penner, R. (1987) J. Physiol. 386, 455–463.
[11] Rehm, H. and Betz, H. (1982) J. Biol. Chem. 257, 10015–10022.
[12] Rehm, H. and Betz, H. (1984) J. Biol. Chem. 259, 6865–6869.
[13] Rehm, H. and Betz, H. (1983) EMBO J. 2, 1119–1122.
[14] Harvey, A.L. and Karlsson, E. (1982) Br. J. Pharmacol. 77, 153–161.
[15] Halliwell, J.V., Othman, I.B., Pelchen-Matthews, A. and Dolly, J.O. (1986) Proc. Natl. Acad. Sci. USA 83, 493–497.
[16] Habermann, E. (1972) Science 177, 314–322.
[17] Stansfeld, C.E., Marsh, S.J., Parcej, D.N., Dolly, J.O. and Brown, D.A. (1987) Neuroscience 23, 893–902.
[18] Schmidt, R.R., Betz, H. and Rehm, H. (1988) Biochemistry 27, 963–967.
[19] Schmidt, R.R. (1988) PhD Thesis, University of Heidelberg.
[20] Bruns, R.F., Lawson-Wendling, K. and Pugsley, T.A. (1983) Anal. Biochem. 132, 74–81.
[21] Clark, S. (1984) in: Receptor Purification Procedures, pp.149–161, A.R. Liss, New York.
[22] Wessel, D. and Flügge, U.I. (1983) Anal. Biochem. 138, 141–143.
[23] McQuarrie, C., Salvaterra, P.M., De Blas, A., Routes, J. and Mahler, H.R. (1976) J. Biol. Chem. 251, 6335–6339.
[24] Caratsch, C.G., Miledi, R. and Strong, P.N. (1985) J. Physiol. 363, 169–179.
[25] Pfeiffer, F., Graham, D. and Betz, H. (1982) J. Biol. Chem. 257, 9389–9393.
[26] Black, A.R. and Dolly, J.O. (1986) Eur. J. Biochem. 156, 609–617.
[27] Rehm, H. and Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. 153, 231–240.
[28] Rehm, H. and Lazdunski, M. (1988) Proc. Natl. Acad. Sci. USA 85, 4919–4923.
[29] Baumann, A., Grupe, A., Ackermann, A. and Pongs, O. (1988) EMBO J. 7, 2457–2463.
[30] Cheng, Y.-C. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099–3108.