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GTP-binding proteins in luminal and basolateral membranes from pars convoluta and pars recta of rabbit kidney proximal tubule

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The GTP-binding proteins on luminal and basolateral membrane vesicles from outer cortex (pars convoluta) and outer medulla (pars recta) of rabbit proximal tubule have been examined. The membrane vesicles were highly purified, as ascertained by electron microscopy, by measurements of marker enzymes, and by investigating segmental-specific transport systems. The [^{35}S]GTPγS binding to vesicles, and to sodium cholate-extracted proteins from vesicles, indicated that the total content of GTP-binding proteins were equally distributed on pars convoluta, pars recta luminal and basolateral membranes. The membranes were ADP-ribosylated with [^{32}P]NAD$^+$ in the presence of pertussis toxin and cholera toxin. Gel electrophoresis revealed, for all preparations, the presence of cholera toxin [^{32}P]ADP-ribosylated 42 and 45 kDa $G\alpha_s$ proteins, and pertussis toxin [^{32}P]ADP-ribosylated 41 kDa $G\alpha_{i1}$, 40 kDa $G\alpha_{i2}$ and 41 kDa $G\alpha_{i3}$ proteins. The 2D electrophoresis indicated that G_o's were not present in luminal nor in basolateral membranes of pars convoluta or pars recta of rabbit proximal tubule.

G-protein; Proximal tubule

1. INTRODUCTION

In renal proximal tubular cells a variety of hormones and neurotransmitters act via membrane receptors coupled to G-proteinse.g. locally, dopamine inhibits the activity of the Na^+/K^+ pump in kidney proximal tubule [1–3], and angiotensin II regulates tubular epithelial transport through receptor-coupled G-proteins [4]. Recently it has been demonstrated that peptide YY equilibrium-binding to basolateral membrane vesicles decreased in a dose-dependent manner in the presence of guanine nucleotides, indicating that the PYY receptors on rabbit proximal tubules are linked to a G-protein [5]. In previous papers we have demonstrated K^+ and Na^+ ion channels in membrane vesicles isolated from outer cortex (pars convoluta) and outer medulla (pars recta) of rabbit proximal tubule [6–9].

In order to understand the functional associations of the various GTP-binding proteins with the different transduction systems we have examined the contents of these proteins in highly purified luminal and basolateral membrane vesicles from pars convoluta and pars recta of rabbit proximal tubule.

Abbreviations: CTX, pertussis toxin; CT, cholera toxin; BBM, brush border membranes; BLM, basolateral membranes.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]NAD$^+$ (10–20 Ci/mmol) was obtained from Amersham, England. [^{35}S]GTPγS was purchased from New England Nuclear. Cholera toxin was from Sigma, USA, while Pertussis toxin was kindly provided by P. Ibsen and I. Heron, Statens Seruminstitut, Denmark.

2.2. *Preparation of renal membrane vesicles*

Luminal membrane vesicles and basolateral membrane vesicles were isolated from either pars convoluta (outer cortex) or pars recta (outer medulla) of the proximal tubule of rabbit kidney according to the method previously described [10] and mentioned here only briefly. Outer cortical tissue was obtained by taking slices <0.3 mm thick from the surface of kidney containing pars convoluta. Strips of outer medulla tissue approx. 1 mm thick (representing predominantly pars recta) were dissected from the outer stripe of the outer medulla. Membrane vesicles from outer cortical and outer medullary tissue were always prepared from the same kidneys, and the two preparations were performed in parallel by using the Ca^{2+} precipitation procedure to separate luminal membranes from basolateral membranes [10]. The vesicles were suspended in a solution containing 310 mm mannitol and 15 mm HEPES/Tris buffer, pH 7.5, and stored in aliquots of 300 μl (30 mg/ml) at −80°C, if not used at once. The purity of the membrane vesicle preparations were examined by electron microscopy and by measuring specific activities of various enzyme markers, as previously described [11]. The amount of protein was determined by the method of Lowry et al. [12] as modified by Peterson [13], with serum albumin (Sigma Chemical Co.) as a standard. All solutions used in this study were sterilized before use.

2.3. *Extraction of proteins*

The vesicles were washed with TED buffer (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DDT, 1 mM PMSF) containing 100 mM NaCl and collected by centrifugation at 19,000 × *g* for 30 min. The washed membranes were suspended in TED buffer, 100 mM NaCl and 2.5% (w/v) sodium cholate, and incubated with stirring on ice for 1 h. Unsolubilized membranes were removed by centrifugation at 100,000 × *g* in an airfuge (175 μl/tube) for 40 min. The supernatant (solubilized

proteins) was assayed for protein content, which was generally 60% of the original material.

2.4. *Binding assays*

The GTP-binding proteins were identified by their ability to bind [^{35}S]GTPγS [14]. The vesicles were washed with TED buffer containing 100 mM NaCl and collected by centrifugation at 19,000 × *g* for 30 min. The protein solution was diluted to a concentration of 5 mg/ml with assay buffer (10 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol 12A9). In a standard assay 90 μl of diluted protein was mixed with 510 μl of incubation medium (100 mM HEPES, pH 8.0, 80 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 400 mM NaCl) containing 0–20 μM [^{35}S]GTPγS (approx. 2 nM, giving 10^5 cpm). After incubation for 30 min at 0°C, 400 μl of the suspension was filtered through a 0.45 μm nitrocellulose filter (Millipore HA-type), which was washed three times with 2 ml of stop solution (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$). The filters were dried for 30 min and solubilized in 5 ml Filter Count (Packard Instrument International SA, Zürich, Switzerland). Radioactivity was counted in a liquid scintillation counter. In case of solubilized protein (see above) the protein was diluted to a concentration of 1 mg/ml with Lubrol buffer (10 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol 12A9). In a standard assay, 20 μl of diluted sample was mixed with 20 μl incubation medium containing [^{35}S]GTPγS. The amount of bound [^{35}S]GTPγS was determined by the nitrocellulose filtration technique as described.

2.5. *ADP ribosylation of G-proteins*

Cholera toxin-dependent ADP ribosylation was carried out by incubating vesicles containing approx. 200 μg protein in 100 μl 100 mM phosphate buffer, pH 7.5, containing 20 mM thymidine, 0.1 mM GTP, 25 μM [^{32}P]NAD^+ (spec. act. 10–20 Ci/mmol) and 2 mM DTT, with and without 10 μg activated (20 mM DTT for 20 min at 30°C) cholera toxin for 30 min at 37°C [15].

Pertussis toxin-dependent ADP ribosylation was accomplished by incubating 50 μg protein 30 min at 37°C in 50 μl 50 mM Tris buffer (pH 7.6) containing 1 mM ATP, 20 mM DTT, 10 mM thymidine, 1 mM EDTA and 10 μM [^{32}P]NAD^+, with and without 5 μg activated pertussis toxin [15].

The reactions were stopped by adding 1 ml ice-cold Tris buffer. Pellets were obtained by centrifugation at 11,000 × *g* for 20 min. The pellets were re-washed and dissolved in Laemnli buffer for PAGE electrophoresis.

2.6. *Electrophoresis*

One-dimensional gel electrophoresis was performed on 15% polyacrylamide gels with 5% stacking gels. Two-dimensional gel electrophoresis was accomplished by the method of O'Farell et al., [16] as modified by Bravo et al. [17]. Pellets from the ADP ribosylation were solubilized in 20 μl lysis buffer (9.8 M urea, 2% (w/v) Nonidet (NP-40), 100 mM DTT, 2% (v/v) Servalytes (pH range 7–9)). Ampholines in the pH range 3.5–10.0 (Pharmacia, Uppsala) and Servalytes (Serva, Heidelberg), pH range 5–7, were used to form a pH 4–8 gradient in the first-dimensional tube gel (4% polyacrylamide gel; acrylamide:bisacrylamide 30:1.7). The second dimension was run in a 15% polyacrylamide gel (acrylamide:bisacrylamide 30:0.15) with a 5% stacking gel. Standards were from Pharmacia, Uppsala (Electrophoresis Calibration Proteins). The gels were dried, and ADP-ribosylated proteins were detected on Amersham film (HYPERfilm-MP).

2.7. *Binding parameters*

The general stoichiometric binding equation may be formulated as:

$$r = \frac{K_1c+2K_1K_2c^2+.....+NK_1K_2....K_Nc^N}{1+K_1c+K_1K_2c^2+.....+K_1K_2....K_Nc^N} \quad (1)$$

where r=average number of moles of GTPγS bound per mole of protein ($(GTP\gamma S)_{bound}/(Pr)_{total}$); c=equilibrium concentration of GTPγS ([GTPγS]); K_j=stoichiometric binding constant for step j [18,19]. The equation can be transformed into:

$$r = \sum_{j=1}^{N} \frac{cK_j}{1+cK_j} \quad (2)$$

In the present work we restricted the problem to decide whether the data could be described by a one-site or multi-site model. Simplifying to a 2 term equation equivalent to a two sites model the equation is:

$$GTP\gamma S_{bound} = \frac{n_1(Pr)_{total}K_1[GTP\gamma S]}{1+K_1[GTP\gamma S]} + \frac{n_2(Pr)_{total}K_2[GTP\gamma S]}{1+K_2[GTP\gamma S]} \quad (3)$$

Equivalent to:

$$GTP\gamma S_{bound} = \frac{B_{max,1}[GTP\gamma S]}{K_{diss,1}+[GTP\gamma S]} + \frac{B_{max,2}[GTP\gamma S]}{K_{diss,2}+[GTP\gamma S]} \quad (4)$$

where K_{diss} = stoichiometric dissociation constants. In the case of 1 site (n=1):

$$GTP\gamma S_{bound} = \frac{B_{max}[GTP\gamma S]}{K_{diss}+[GTP\gamma S]} \quad (5)$$

The binding parameters were determined by using non-linear regression [20]. The equations (one-site model or two-sites model) were fitted to the data by finding the parameters, i.e. B_{max}, K_{diss} (eqn. 5) or $B_{max,1}$, $K_{diss,1}$, $B_{max,2}$, $K_{diss,2}$ (eqn. 4) that cause the equations to best fit the data. The norm represents the closeness of the fit of the iteration: numerically it is the square root of the sum of squares of the residuals.

3. RESULTS AND DISCUSSION

3.1. *Purity of membranes*

During the preparation procedure a number of marker enzymes characteristic for the luminal- and the basolateral membranes were measured. The Na^+/K^+-ATPase activity was enriched by a factor of 15.7 in basolateral membrane vesicles compared to the crude cortical homogenate, while the activity was de-enriched in luminal membrane vesicles (by a factor of 0.6). The luminal membrane marker enzymes, alkaline phosphatase and trehalase, were enriched 13-fold in the luminal membrane vesicles compared to the crude homogenate. In basolateral membrane vesicles the enrichment for these marker enzymes was a factor of 1.5 and 1.8, respectively. The results showed that the luminal membranes contained only approx. 4% of basolateral membrane proteins, while the basolateral membranes were contaminated with approx. 10% of luminal membrane proteins. Previously we have demonstrated multiple α-amino acid–Na^+ co-transport systems in luminal membranes from pars convoluta or pars recta vesicles with different characteristics given by V_{max} and K_{diss} [21–27]. Therefore, the preparation procedure was further examined by measuring several of these transport systems specific for the distinct regions of the proximal tubule. As described above several steps were used in the preparation procedure to remove intracellular organelles. However, minor contaminations of subcellular structures could not be excluded.

3.2. *[^{35}S]GTPγS binding properties*

The vesicles and solubilized vesicles were analyzed for their ability to bind [^{35}S]GTPγS to obtain a measure of the total amount of GTP-binding proteins in the membranes.

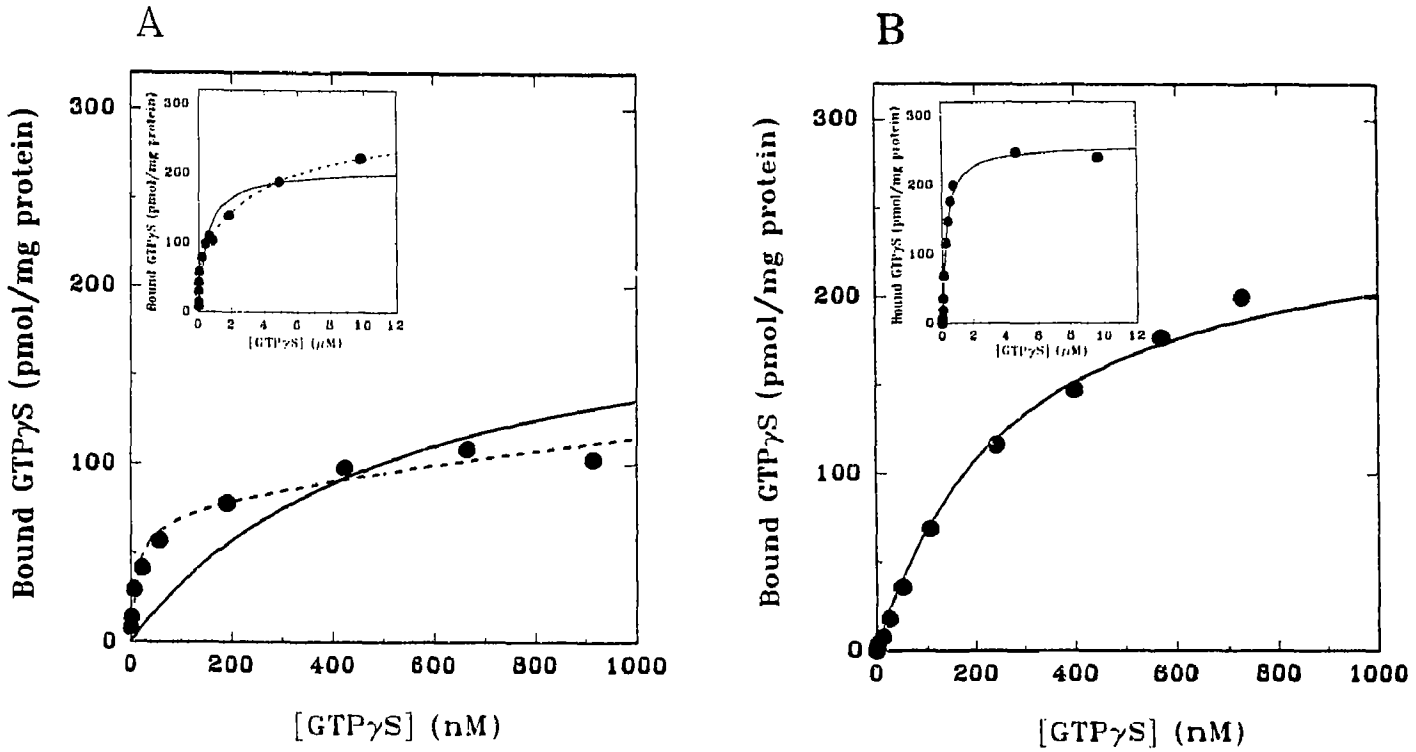

Fig. 1. Binding data approximated by using least-squares non-linear regression to a one-site (eqn. 5, solid lines) or several (two)-sites binding model (eqn. 4, dotted lines). Plots of the full range (up to 12 μM) are inserted in each figure. (A) [^{35}S]GTPγS binding measured to luminal vesicles isolated from pars recta. (B) Luminal vesicles from pars recta solubilized in sodium cholate.

Fig. 1 presents examples of binding data approximated by using least-squares non-linear regression to a one-site (eqn. 5) or a two-sites binding model (eqn. 4). The [^{35}S]GTPγS binding was measured to vesicles (Fig. 1A) and to vesicles solubilized in sodium cholate (Fig. 1B). Plots in the full range (up to 12 μM) are inserted in each figure. It should be noted that full-range data were used for calculation of binding parameters. As we studied the GTP-binding proteins in an isolated system we chose to work with concentrations of Mg^{2+} in the millimolar range ([Mg^{2+}]>20 mM), which gave maximal [^{35}S]GTPγS binding (pilot experiments, data not shown). Under these conditions receptor-stimulated GTP-binding proteins was shown to bind maximal amounts of [^{35}S]GTPγS even without receptor stimulation [28]. It can be seen thatm the binding data in Fig. 1A, luminal pars recta vesicles, are best described by a several (two)-sites model (dotted lines), whereas the solubilized vesicles binding data in Fig. 1B may be described by either equation. The binding parameters for all membranes are summarized in Table I. The one-site approximation constant, B_{max}, should give a measure of the total amount of G-proteins in the distinct proximal tubule membrane vesicles. As seen from the table, the B_{max} values are comparable for pure vesicles and solubilized vesicles from the four separate regions. However, B_{max} for intact pars convoluta luminal vesicles are lower than the others, perhaps because [^{35}S]GTPγS is not easily penetrating these vesicle membranes. On average, 200 pmol [^{35}S]GTPγS could be bound per mg of protein. As assessed by the norm values of eqn. 5 and eqn. 4 in Table I the binding data were best fitted by a multi-site model, i.e. the two-terms binding equation. Fig. 1A and Table I clearly revealed that high-affinity binding is retained in intact vesicles, since we obtained $K_{diss,1}$ values at about 20 nM. These results are comparable to the $K_{d(app)}$ values of 50–100 nM reported for G_i-proteins [29]. Fitting of the data to the two-terms binding equation reveals that the solubilization procedure had a profound effect on the GTP binding. The measured $K_{diss,1}$ values were in the order of ten-fold lower for the vesicles than for the solubilized proteins. Obviously the detergent disturbed the binding of [^{35}S]GTPγS, either by competitive interactions or by changing the local environment around the GTP binding site.

3.3. *Pertussis- and cholera toxin-catalyzed ADP ribosylation*

In order to characterize the distribution of heterotrimeric G-proteins toxin-catalyzed ribosylation was performed. Fig. 2A shows the one-dimensional gel electrophoresis of [^{32}P]NAD$^+$ ADP-ribosylated G-proteins. As expected the distribution of G-proteins in the various segments of the kidney proximal tubule is heterogeneous. In PTX ribosylations, (lanes 1–4) strong incorporation of [^{32}P]ADP-ribosyl is found in proteins of

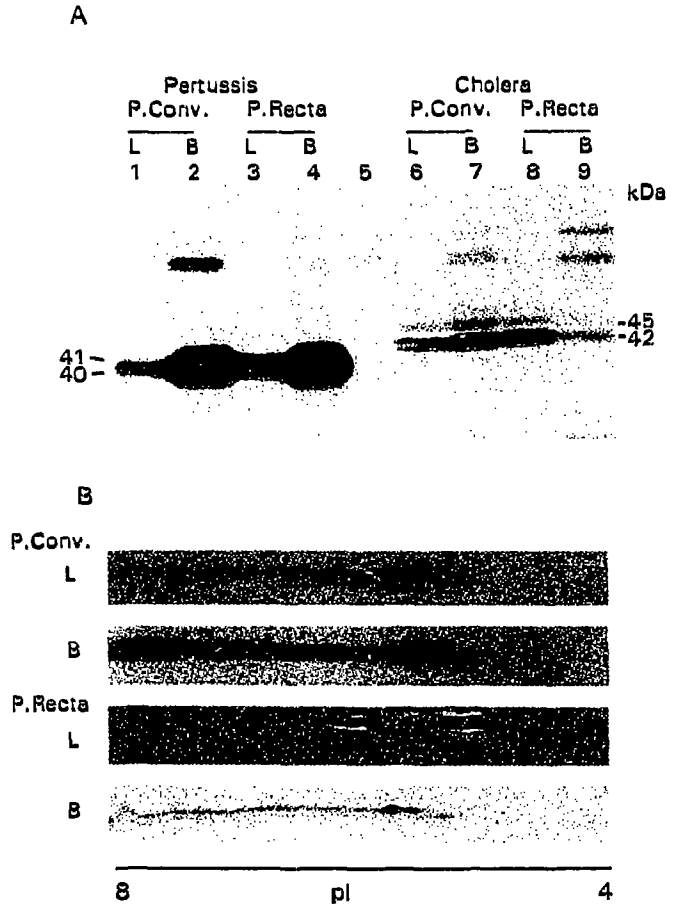

Fig. 2. One- and two-dimensional gel electrophoresis of [^{32}P]NAD$^+$ ADP-ribosylated G-proteins. (A) One-dimensional. (B) Two dimensional.

40–41 kDa, especially in basolateral membranes (lanes 2 and 4). Lane 5 shows a reference, pars recta basolateral vesicles treated in the absence of toxin. The high molecular weight proteins that are seen in lanes 2, 4, 6 and 8 are also seen here. The apparent lower level of PTX ribosylation in luminal membranes might partially be explained by the orientation of the vesicles. Electron microscopical studies of the vesicles showed that the absolute majority of the luminal vesicles were the 'right-side out' and are thus perhaps less accessible to ADP ribosylation, while about 50% of the basolateral vesicles had the 'inside-out' orientation and were probably more easily accessible [30]. Cholera toxin [^{32}P]ADP ribosylates a 42 and 45 kDa protein in both luminal- and basolateral membranes (Fig. 2A, lanes 6–9). These CTX ADP-ribosylated G-proteins might represent different forms of the classical $G_{s\alpha}$ subunit, or the 42 kDa protein could be a new CTX substrate, as described by Zhou et al. [31].

To further identify the PTX [^{32}P]NAD$^+$ ADP-ribosylated G-proteins we performed two-dimensional gel electrophoresis for each anatomical location. It appears from Fig. 2B that PTX [^{32}P]ADP-ribosylated 40 and 41 kDa G-proteins were split in three different spots. All samples from the four different membranes exhibit the same two-dimensional pattern, although with different intensities of the spots. The broad 40–41 kDa band from the one-dimensional gel could be resolved into three spots (apparent pI values of 5.3, 5.6 and 5.8). The two most basic proteins had a mol. wt. of 41 kDa, while the most acidic protein was determined as being 40 kDa. However, Fig. 2B reveals that no 39 kDa proteins were PTX ribosylated. This seems to indicate that no G_o's are present in luminal nor in basolateral membranes from pars convoluta or pars recta of rabbit proximal tubule, a finding which is in agreement with that in bovine kidney cortex [32] and in cortical tubular cells form rat kidney [30]. Thus we may conclude that the three PTX-ribosylated proteins are $G\alpha_{i1}$ (41 kDa), $G\alpha_{i2}$ (40 kDa) and $G\alpha_{i3}$ (41 kDa).

Table I

Binding parameters obtained by using nonlinear regression [20] of equations 5 and 4 to the experimental [^{35}S]GTPγS binding data

	$B_{max.}$ (pmol/mg)	$K_{diss.}$ (nM)	$B_{max.1}$ (pmol/mg)	$K_{diss.1}$ (nM)	$B_{max.2}$ (pmol/mg)	$K_{diss.2}$ (mM)	Norm (eqn. 5)	Norm (eqn. 4)
Vesicles								
PCT								
luminal	120	1052	30	25	102	3,191	34.3	9.6
basolateral	210	371	89	19	188	3,372	72.3	12.4
PST								
luminal	207	529	73	14	208	3,883	77.0	18.6
basolateral	234	497	82	12	216	3,049	85.0	28.0
Solubilized Vesicles								
PCT								
luminal	211	944	94	299	165	3,971	17.4	7.1
basolateral	238	289	147	86	132	2,925	44.7	14.5
PST								
luminal	258	280	120	280	138	280	20.5	20.5
basolateral	111	364	12	324	99	369	8.4	8.4

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REFERENCES

[1] Birnbaumer, L., Abramowitz, J. and Brown, A.M. (1990) Biochim. Biophys. Acta 1031, 163–224.
[2] Bertorello, A. and Aperia, A. (1989) Am. J. Physiol. 256, F57–62.
[3] Felder, C.C., McKelvey, A.M., Gitler, M.S., Eisner, G.G. and Jose, P.A. (1989) Kidney Int. 36, 183–193.
[4] Douglas, J.G. (1987) Am. J. Physiol. 22, F1–F7.
[5] Sheikh, S.P., Sheikh, M.I. and Schwartz, T.W (1989) Am. J. Physiol. 257, F978–F984.
[6] Jacobsen, C., Røigaard-Petersen, H. and Sheikh, M.I. (1989) Biochem. J. 262, 271–275.
[7] Jacobsen, C., Røigaard-Petersen, H. and Sheikh, M.I. (1989) FEBS Lett. 243, 173–176.
[8] Jacobsen, C., Røigaard-Petersen, H. and Sheikh, M.I. (1988) FEBS Lett. 236, 95–99.
[9] Blokkebak-Poulsen, J., Sheikh, M.I. and Jacobsen, C. (1990) Biochem. J. 272, 839–842.
[10] Sheikh, M.I. and Møller, J.V. (1987) In: Biochemical Toxicology: A Practical Approach (Snell, K. and Mullock, B., eds.) Ch. 7, pp. 153–182, IRL Press, Oxford.
[11] Kragh-Hansen, U. and Sheikh, M.I. (1984) J. Physiol. 354, 55–67.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[14] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[15] Bellorin-Font, E., Starosta, R., Milanes, C.L., Lopez, C., Pernalete, N., Weisinger, J. and Paz-Martinez, V. (1990) Am. J. Physiol. 258, F1640–F1649.
[16] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[17] Bravo, R. (1984) in: Two Dimensional Gel Electrophoresis of Proteins: Methods and Applications (Celis, J.E. and Bravo, R., eds.) Acadademic Press, New York.
[18] Adair, G.S. (1925) J. Biol. Chem. 63, 529–545.
[19] Klotz, I.M. (1946) Arch. Biochem. 9, 109–117.
[20] Draper, N.R. and Smith, H. (1981) Applied Regression Analysis, 2nd edn., pp. 458–529, Wiley, New York.
[21] Kragh-Hansen, U., Røigaard-Petersen, H., Jacobsen, C. and Sheikh, M.I. (1984) Biochem. J. 220, 15–24.
[22] Jørgensen, K.E. and Sheikh, M.I. (1987) Biochem. J. 248, 533–538.
[23] Røigaard-Petersen, H., Jacobsen, C. and Sheikh, M.I. (1987) Am. J. Physiol. 253, F15–F20.
[24] Røigaard-Petersen, H., Jacobsen, C. and Sheikh, M.I. (1988) Am. J. Physiol. 254, F628-F633.
[25] Vorum, H., Jessen, H., Jørgensen, K.E. and Sheikh, M.I. (1988) FEBS Lett. 227, 35–38.
[26] Jessen, H., Jørgensen, K.E., Røigaard-Petersen, H. and Sheikh, M.I. (1989) J. Physiol. 411, 517–528.
[27] Røigaard-Petersen, H., Jessen, H., Mollerup, S., Jørgensen, K.E., Jacobsen, C. and Sheikh, M.I. (1990) Am. J. Physiol. 258 (1990) F388-F396.
[28] Iyengar, R. and Birnbaumer, L. (1982) Proc. Natl. Acad. Sci. USA 79, 5179–5183.
[29] Carty, D.J., Padrell, E., Codina, J., Birnbaumer, L., Hildebrandt, J.D. and Iyengar, R. (1990) J. Biol. Chem. 265, 6268–6273.
[30] Van der Ploeg, I., Fredholm, B.B., Carlquist, M.M., Bertorello, A., Aperia, A. and Celsi, G. (1991) Acta Physiol. Scand. 142, 83–90.
[31] Zhou, J., Sims, C., Chang, C.H., Berti-Mattera, L., Hopfer, U. and Douglas, J. (1990) Proc. Natl. Acad. Sci. USA 87, 7532–7535.
[32] Murakami, T., Rossiter, K., Spiegel, A.M. and Sactor, B. (1989) Biochem. Pharmacol. 38, 4502–4505.