The soluble 'low- K_m ' 5'-nucleotidase of rat kidney represents solubilized ecto-5'-nucleotidase

Grazyna PIEC and Michel LE HIR*

Departement Forschung, Kantonsspital, CH-4031 Basel, Switzerland

A soluble 'low- K_m ' 5'-nucleotidase has been described previously in several organs. It has been presumed to be of cytosolic origin and thus to play a role in the intracellular production of adenosine. Its catalytic properties are similar to those of the ecto-5'-nucleotidase of cell membranes. In the present study we compared molecular properties of the two enzymes in the kidney of the rat. The M_r of the main peak of soluble 'low- K_m ' 5'-nucleotidase in gel-filtration chromatography was similar to that of the ecto-5'-nucleotidase solubilized by a phosphatidylinositol-specific phospholipase C from renal brush-border membranes. In phase-partition experiments using Triton X-114, the soluble enzyme appeared to be hydrophobic. Its hydrophobicity was decreased on treatment with a phosphatidylinositol-specific phospholipase C, suggesting that the soluble 'low- K_m ' 5'-nucleotidase contains the phosphatidylinositol anchor which is characteristic for the ecto-enzyme. An anti-ecto-5'-nucleotidase antiserum provoked an almost complete inhibition of the soluble enzyme. Immunoblotting using anti-ecto-5'-nucleotidase antiserum revealed in the high-speed supernatants a polypeptide with a similar M_r to the subunit of the ecto-5'-nucleotidase. The soluble 'low- K_m ' 5'-nucleotidase, like the ecto-5'-nucleotidase, bound specifically to concanavalin A. We conclude that the soluble 'low- K_m ' 5'-nucleotidase is not a cytosolic enzyme, but that it most probably originates from the solubilization of the ecto-5'-nucleotidase, and that it therefore cannot participate in the intracellular production of adenosine.

INTRODUCTION

Adenosine exerts various regulatory functions via specific membrane receptors in many cell types. It originates mainly from the hydrolysis of 5'-AMP by 5'-nucleotidase (Newby, 1984). Three types of 5'-nucleotidases showing activity with 5'-AMP have been extensively described in several organs of mammals. One is anchored at the extracellular surface of the cell membrane, and therefore it is known as the ecto-5'-nucleotidase. The two other types of 5'-nucleotidase are soluble, and therefore possibly cytosolic, enzymes. From a physiological point of view, an enzyme producing adenosine in the cytosol seems more important than the ecto-5'-nucleotidase, which only hydrolyses extracellular 5'-AMP. Actually, it is well known that the rate of production of adenosine is strongly influenced by cellular metabolism and in particular by the intracellular concentration of ATP (Newby, 1984). The two types of soluble 5'-nucleotidases are very different from each other. As one shows K_m values for 5'-AMP in the micromolar range and those for the other are in the millimolar range, it seems appropriate to call them respectively soluble 'low- K_m ' 5'-nucleotidase and soluble 'high- K_m ' 5'-nucleotidase (Spychala *et al.*, 1989). The effects of various natural regulators on these two enzymes are strikingly different. ATP stimulates the soluble 'high- K_m ' 5'-nucleotidase (Van den Berghe *et al.*, 1977; Itoh, 1981), but inhibits the soluble 'low- K_m ' 5'-nucleotidase (Madrid-Marina & Fox, 1986; Le Hir & Dubach, 1988). The former is completely inactive unless Mg^{2+} is added to the incubation medium (Itoh, 1981), whereas the latter can be assayed without addition of divalent cations (Mallol & Bozal, 1983; Fritzon *et al.*, 1986; Le Hir & Dubach, 1988). Maximal activity is obtained in the acidic range for the soluble 'high- K_m ' 5'-nucleotidase (Itoh, 1981) and in the alkaline range for the soluble 'low- K_m ' 5'-nucleotidase (Montero & Fes, 1982; Fritzon *et al.*, 1986; Madrid-Marina & Fox, 1986; Le Hir & Dubach, 1988). The above-mentioned catalytic properties of the soluble 'low- K_m ' 5'-nucleotidase are shared also by the ecto-5'-nucleo-

tidase. In addition, the two enzymes show structural similarities. An M_r value of the subunit of about 70000 has been found for the soluble enzyme from placenta (Madrid-Marina & Fox, 1986) as well as for the ecto-enzyme in various tissues (Harb *et al.*, 1983; Dieckhoff *et al.*, 1985; Naito & Lowenstein, 1985; Thompson *et al.*, 1987; Le Hir *et al.*, 1989). The soluble enzyme from placenta (Madrid-Marina & Fox, 1986) and from rat liver (Fritzon *et al.*, 1986) as well as the ecto-enzyme from various sources (Slavik *et al.*, 1977; Fini *et al.*, 1986; Thompson *et al.*, 1987; Le Hir *et al.*, 1989) specifically bind to concanavalin A.

Considering the number of common properties of the ecto-5'-nucleotidase and of the soluble 'low- K_m ' 5'-nucleotidase, the question arises of whether one might be dealing with two forms of the same enzyme. The results of the present study suggest an affirmative answer to this question for these enzymes in rat kidney.

MATERIALS AND METHODS

Animals

We used male Wistar rats of 200–250 g body wt.

Chemicals

5'-AMP was obtained from Boehringer (Mannheim, Germany), 5'-[U- ^{14}C]AMP was from Amersham International (Amersham, Bucks., U.K.), Sephacryl S-300 and concanavalin A–Sephacryl were from Pharmacia (Uppsala, Sweden), Nembutal was from Abbot Laboratories (Zug, Switzerland), and phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (PIPLC) was from Pharmacia (Brugg, Switzerland).

Assay of 5'-nucleotidase

The incubation medium consisted of 50 μM 5'-AMP, tracer amounts of 5'-[U- ^{14}C]AMP, 2 mM β -glycerophosphate and 0.02 % sulphobetaine 14 in 20 mM-Pipes buffer titrated to pH 7.6 with NaOH at 37 °C. The incubation was for 30 min at 37 °C.

Abbreviations used: PIPLC, phosphatidylinositol-specific phospholipase C.

* To whom correspondence should be addressed.

Labelled adenosine produced by hydrolysis of labelled 5'-AMP was determined as described by Panagia *et al.* (1986).

Preparation of the high-speed supernatants

In order to wash the blood out, the kidneys of rats anaesthetized with nembutal (100 mg/kg body wt.) were perfused *in situ* via the aorta with 25 ml of a buffer consisting of 25 mM-Tris, 150 mM-NaCl, 2 mM-MgCl₂ and 1 mM-EDTA, titrated to pH 7.6 with HCl. The perfusion solution was at room temperature, and the perfusion time did not exceed 10 s. The kidneys were removed immediately, minced with a razor blade, and homogenized by 10 strokes at 1000 rev./min in a Potter homogenizer with a Teflon pestle (clearance 0.1 mm). The buffer for homogenization was the same as that used for perfusion; 3 ml/g of tissue was used. The homogenization and all subsequent procedures took place at 4 °C. The homogenate was centrifuged successively at 2500 g for 15 min, at 18000 g for 15 min and at 100000 g for 60 min.

Preparation of solubilized ecto-5'-nucleotidase from brush-border membranes

Renal brush-border membranes were prepared by MgCl₂ precipitation (Biber *et al.*, 1981). Brush-border membranes (1 mg of protein/ml) were incubated with PIPLC (0.7 µg/ml) for 30 min at 25 °C in 20 mM-Mops/150 mM-sucrose/50 mM-NaCl titrated to pH 7.4 with NaOH. The incubation mixture was centrifuged at 100000 g for 60 min.

Gel-filtration chromatography

This was carried out at 4 °C at a flow rate of 15 ml/h; 3 ml of high-speed supernatant was applied on to a Sephacryl S-300 column (2 cm × 70 cm) equilibrated with a buffer consisting of 20 mM-Tris, 300 mM-NaCl and 1 mM-MgCl₂, titrated to pH 7.6 with HCl. The column was calibrated with a mixture of *M_r* standards (Bio-Rad Laboratories, Richmond, CA, U.S.A.), consisting of bovine thyroglobulin (*M_r* 670000), bovine catalase (*M_r* 235000), bovine lactate dehydrogenase (*M_r* 140000), and human haemoglobin (*M_r* 64500). In some instances the samples were treated with 0.1 % of Triton X-100 for 30 min at 4 °C before chromatography, and 0.1 % of the detergent were added to the buffer for equilibration of the column.

Concanavalin A-Sepharose chromatography

Chromatography was carried out at 22 °C; 3 ml of high-speed supernatant of renal homogenates was applied on to a concanavalin A-Sepharose column (1 cm × 2 cm) equilibrated with a buffer consisting of 20 mM-Tris, 500 mM-NaCl, 1 mM-MnCl₂, 1 mM-MgCl₂ and 1 mM-CaCl₂, titrated to pH 7.6 with HCl. The flow rate was 14 ml/h during application of the sample and wash and 4 ml/h during elution. After washing with 2 ml of the buffer, elution was performed with 400 mM-methyl α-D-mannopyranoside in the buffer.

Phase partition with Triton X-114

A 4 ml portion of the sample containing 5'-nucleotidase activity was mixed at 4 °C with 0.2 ml of a precondensed 20 % solution of Triton X-114. The separation of the phases was performed as described by Bordier (1981), except that the buffer phase was not washed, to minimize the loss of enzymic activity.

Effect of antibodies on the activity of 5'-nucleotidase

The high-speed supernatants of rat renal homogenates, diluted 1:100, were incubated for 1 h at 4 °C in a volume ratio of 1:1 with various dilutions of an anti-(rat ecto-5'-nucleotidase) rabbit antiserum (Dawson *et al.*, 1989) or of the pre-immune rabbit serum. The buffer used for all dilutions consisted of phosphate-

buffered saline (137 mM-NaCl, 2.7 mM-KCl, 8.8 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, 0.9 mM-CaCl₂, 0.5 mM-MgCl₂) containing 0.1 % bovine serum albumin.

Immunoblotting

Purified ecto-5'-nucleotidase from rat kidney (Le Hir *et al.*, 1989) and high-speed supernatants of rat kidney homogenates were submitted to SDS/PAGE under reducing conditions as described by Laemmli (1970). The proteins were electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979). Incubation of the nitrocellulose sheets with rabbit anti-5'-nucleotidase antiserum and detection of the bound rabbit immunoglobulins were carried out as described by Dawson *et al.* (1989).

RESULTS

As the assays for 5'-nucleotidase in the present study were done with 50 µM-5'-AMP and in the absence of bivalent cations, the 'high-*K_m*' 5'-nucleotidase cannot have contributed to the measured activities (Itoh, 1981). β-Glycerophosphate was included in the incubation medium in order to minimize the hydrolysis of 5'-AMP by non-specific phosphatases. Under such conditions of assay, neither tartrate, an inhibitor of acid phosphatase, nor levamisole, an inhibitor of alkaline phosphatase, had any effect on the hydrolysis of 5'-AMP by high-speed supernatants of rat kidney homogenates. The measured activities can thus be attributed to the 'low-*K_m*' 5'-nucleotidase.

One, two, five and 15 strokes of the Teflon pestle released

Fig. 1. Gel-filtration chromatography on Sephacryl S-300 of the high-speed supernatant of a rat kidney homogenate in the absence (a) and in the presence (b) of 0.1 % Triton X-100

The elution volumes of *M_r* markers are indicated by numbered vertical bars: 1, void volume; 2, thyroglobulin; 3, catalase; 4, lactate dehydrogenase; 5, haemoglobin.

4.5 %, 5.6 %, 6.2 % and 6.8 %, respectively, of the total activity of 5'-nucleotidase of the homogenate of rat kidney. With the ten strokes used routinely we found an activity of $1.53 \pm 0.26 \mu\text{mol/min per ml}$ in the homogenate. The activity in the high-speed supernatant was $0.13 \pm 0.04 \mu\text{mol/min per ml}$ (means $\pm$ S.D.; $n = 8$), corresponding to 6.7 % of the total activity in the homogenate.

The elution profile in gel-filtration chromatography on Sephacryl S-300 of the 5'-nucleotidase activity of high-speed supernatants of homogenates of rat kidneys (washed free of blood) showed two main peaks of activity (Fig. 1). One peak corresponded to the void volume of the column. An M_r of 310 000 was calculated from the elution volume of the second peak. If the chromatography was carried out in the presence of Triton X-100, then most of the activity was detected in one peak (Fig. 1), for which an M_r of 440 000 was calculated.

When a suspension of renal brush-border vesicles was centrifuged at 100 000 g for 60 min after treatment with PIPLC, more than 90 % of the activity of 5'-nucleotidase was recovered in the high-speed supernatant. Gel-filtration chromatography on Sephacryl S-300 of that supernatant revealed one peak of 5'-nucleotidase activity (results not shown). Its elution volume corresponded to an M_r of 275 000, and this was not affected by the presence of Triton X-100.

High-speed supernatants of renal homogenates, as well as the second peak (M_r 310 000) of activity eluted from the gel-filtration column during chromatography of these supernatants, were subjected to phase partition with Triton X-114. The total recoveries of 5'-nucleotidase activity in the two phases were 95 ± 3 % for the supernatants and 90 ± 7 % for the eluted peak (means $\pm$ S.D., $n = 6$). From the total recovered activity, 23 ± 2 % and 26 ± 3 % ($n = 6$) were in the detergent-poor phases for the supernatant and the eluted peak respectively. If a sample of the 5'-nucleotidase eluted from the gel filtration column was treated with PIPLC, then 77 ± 10 % ($n = 3$) of the activity was recovered in the detergent-poor phase. Of the activity eluted during gel-filtration chromatography of the PIPLC extract of brush-border

Fig. 2. Inhibition of the 'low- K_m ' 5'-nucleotidase in high-speed supernatants of rat renal homogenates by rabbit sera

The high-speed supernatants of rat renal homogenates diluted 1:100 were incubated for 1 h at 4 °C in a volume ratio of 1:1 with various dilutions of a rabbit anti-(rat ecto-5'-nucleotidase) antiserum (—) or of the preimmune rabbit serum (----). The activity is expressed as the percentage of a control without serum.

Fig. 3. Immunoblot of a high-speed supernatant of renal homogenate with an anti-ecto-5'-nucleotidase antiserum

A 10 μl sample of high-speed supernatant of renal homogenate obtained as described in the Materials and methods section (lane A) and 2 ng of purified ecto-5'-nucleotidase (lane B) were submitted to SDS/PAGE under reducing conditions. The nitrocellulose sheets were incubated successively with a rabbit anti-(rat ecto-5'-nucleotidase) antiserum at a dilution of 1:1000, with a peroxidase-conjugated pig anti-(rabbit Ig) antibody, and with a peroxidase-anti-peroxidase complex. Peroxidase activity was revealed with chloronaphthol as the chromogen.

membranes, 96 ± 1 % ($n = 3$) was recovered in the detergent-poor phase.

Preincubation of the high-speed supernatant of the renal homogenate with an antiserum against purified ecto-5'-nucleotidase, but not with pre-immune serum, provoked an almost complete inhibition of the soluble 5'-nucleotidase activity (Fig. 2).

The 5'-nucleotidase activity of high-speed supernatants of renal homogenates was almost complete (98 %) retained on a concanavalin A-Sepharose column, and it could be eluted quantitatively (103 %) by methyl α -D-mannopyranoside.

Immunoblotting of proteins of the high-speed supernatant of rat renal homogenate after separation by SDS/PAGE revealed, at the same position as the purified ecto-5'-nucleotidase, a band reacting with anti-ecto-5'-nucleotidase antiserum (Fig. 3).

DISCUSSION

The cellular site of production of adenosine is still a matter of discussion. Some studies using the heart (Schütz *et al.*, 1981; Meghji *et al.*, 1988) or polymorphonuclear leucocytes (Newby & Holmquist, 1981) led to the conclusion that adenosine is produced mainly intracellularly by a cytosolic 5'-nucleotidase. In other studies it was concluded that adenosine is produced mainly extracellularly by the ecto-5'-nucleotidase in heart (Ribeiro & Sebastiao, 1987; Van Belle *et al.*, 1987; Headrick & Willis, 1989; Imai *et al.*, 1989), in endothelial cells (Shryok *et al.*, 1988) and in kidney (Ramos-Salazar & Baines, 1986). The question of the origin of adenosine is complicated additionally by the existence of two types of soluble, presumably cytosolic, 5'-nucleotidase activities. They have been called soluble 'high- K_m ' 5'-nucleotidase and soluble 'low- K_m ' 5'-nucleotidase (Spychala *et al.*, 1989).

The present study is devoted to the soluble 'low- K_m ' 5'-nucleotidase, which, from the catalytic point of view appears

appropriate for the intracellular production of adenosine. However, although this enzyme has been supposed to be of intracellular origin (Fredholm & Lindgren, 1983; Mallol & Bozal, 1983; Fritzon *et al.*, 1986; Madrid-Marina & Fox, 1986; Le Hir & Dubach, 1988; Spychala *et al.*, 1989), this point has not been definitely established. The catalytic and molecular properties of the soluble 'low- K_m ' 5'-nucleotidase show striking similarities with those of the membrane enzyme ecto-5'-nucleotidase. We therefore suspected that one might be dealing with the same enzyme. The detection of a soluble form of the ecto-enzyme is actually not surprising, since there exist in blood plasma a phospholipase D (Low & Prasad, 1988) and in the liver a phospholipase C (Fox *et al.*, 1987) both capable of splitting specifically the membrane anchor of a group of proteins, among them ecto-5'-nucleotidase, that are anchored to the lipid bilayer of cell membranes by a covalent bond with a phosphatidylinositol-glycan (Low, 1989). Thompson *et al.* (1987) found similarities between the ecto-5'-nucleotidase and the soluble 5'-nucleotidase of the human placenta and proposed that the ecto-enzyme might be released in a soluble form by an endogenous phospholipase C. Five lines of evidence derived from the results of the present study support our suspicion that the soluble 'low- K_m ' 5'-nucleotidase of renal homogenates might originate from the solubilization of the membrane enzyme ecto-5'-nucleotidase of renal homogenates. Firstly, the activity of 'low- K_m ' 5'-nucleotidase in high-speed supernatants of rat kidneys could be inhibited by antibodies directed against the ecto-5'-nucleotidase. Secondly, the 'low- K_m ' 5'-nucleotidase in the high-speed supernatants, like the ecto-5'-nucleotidase, was retained on a concanavalin A column, and it could be eluted by methyl α -D-mannopyranoside. This indicates that the soluble enzyme is a glycoprotein. It can therefore hardly originate from the cytosol. Thirdly, most of the soluble 'low- K_m ' 5'-nucleotidase activity was recovered in the detergent-rich phase in phase-partition experiments using Triton X-114. Most of the activity was recovered in the buffer phase only after treatment with phospholipase C. Thus, at least part of the soluble 'low- K_m ' 5'-nucleotidase is associated with a phosphatidylinositol which probably represents the membrane anchor of the ecto-5'-nucleotidase. Fourthly, the second peak of 'low- K_m ' 5'-nucleotidase eluted from Sephacryl S-300 and the ecto-5'-nucleotidase released from brush-border membrane vesicles by phospholipase C had similar M_r values, as determined by gel-filtration chromatography. The higher M_r calculated for the peak from the supernatants of the homogenates might be due to a shift owing to the larger forms of 5'-nucleotidase, probably aggregates, which are evident in Fig. 1. Fifthly, immunoblotting of the high-speed supernatant using anti-ecto-5'-nucleotidase antiserum revealed one band with a similar subunit M_r to the subunit of the ecto-5'-nucleotidase.

According to its elution pattern in gel-filtration chromatography, the M_r of the main species of soluble 'low- K_m ' 5'-nucleotidase would be about 310 000. Activity eluted before the main peak might represent aggregates occurring by hydrophobic interactions. In fact, when chromatography was carried out in the presence of Triton X-100, then most of the activity was eluted in one peak. An M_r of 280 000 has been calculated for the soluble 'low- K_m ' 5'-nucleotidase from rat liver on the basis of gel-filtration chromatography (Fritzon *et al.*, 1986). As the M_r of the subunit of the ecto-5'-nucleotidase is close to 70 000 (Harb *et al.*, 1983; Dieckhoff *et al.*, 1985; Naito & Lowenstein, 1985; Thompson *et al.*, 1987; Le Hir *et al.*, 1989), the soluble enzyme in the liver and in the kidney of the rat may be constituted of four subunits of the ecto-5'-nucleotidase. In the human placenta, on the contrary, the soluble 'low- K_m ' 5'-nucleotidase appeared as a dimer of M_r 143 000 determined by gel filtration (Madrid-Marina

& Fox, 1986). Solubilization of the ecto-5'-nucleotidase from renal brush-border vesicles with PIPLC yields a molecule of M_r 275 000 as determined by gel-filtration chromatography. It probably represents a tetramer. This contrasts with the dimeric form of alkaline phosphatase released from human placenta by PIPLC, although the native structure of this enzyme also corresponds to a tetramer (Hawrylak & Stinson, 1988).

The proportion (6.8%) of the total activity of 5'-nucleotidase which is found in the high-speed supernatant is higher in rat kidney than in rat liver (1.5%) (Fritzon *et al.*, 1986), and it is particularly high in bovine brain (Montero & Fes, 1982). These differences are not likely to be due to the procedure of homogenization. In the studies mentioned above, liver and brain were both homogenized in Potter homogenizers with 0.25 M-sucrose. In preliminary experiments in the present study, kidneys were homogenized with 0.25 M-sucrose or with buffered saline solutions at pH values between 6.0 and 8.2. The soluble activity was little influenced by the composition of the solution. Madrid-Marina & Fox (1986) did not indicate the proportion of soluble activity in the placenta.

The solubilization of 5'-nucleotidase might be a consequence of the hydrolysis of the anchors by phospholipases or proteases. However, this proposal appears to contradict the finding that in phase-partition experiments with Triton X-114 only 23% of the renal soluble 'low- K_m ' 5'-nucleotidase was recovered in the detergent-poor phase, whereas this phase contained 96% of the ecto-5'-nucleotidase solubilized from brush-border membrane by PIPLC. This suggests that at least some subunits of the renal soluble 'low- K_m ' 5'-nucleotidase present in supernatants of homogenates comprise the phosphatidylinositol-glycan anchor. In fact, treatment of the supernatants with PIPLC resulted in a recovery of 77% of the soluble enzyme in the detergent-poor phase. The effect of Triton X-100 on the behaviour of 5'-nucleotidase of the supernatants of homogenates in gel-filtration chromatography, namely the disappearance of the species of M_r 310 000 and the appearance of a main peak corresponding to M_r 440 000, further suggests that the soluble enzyme contains a hydrophobic moiety. The species of M_r 440 000 might represent a tetramer associated with a micelle of the detergent. An M_r of 90 000 has been reported for a micelle of Triton (Hjelmeland & Chrambach, 1984). The 5'-nucleotidase released by PIPLC from brush-border vesicles behaves in the same way in gel-filtration chromatography with and without detergent, which suggests that the enzyme does not interact with Triton X-100 after removal of the anchor. Thus, if most of all of the soluble 'low- K_m ' 5'-nucleotidase represents ecto-5'-nucleotidase released from the membrane by hydrolysis of the anchor, it must be assumed that this solubilization does not require the hydrolysis of the anchors of all four subunits. After hydrolysis of part of the four anchors of the tetramer, a micro-micelle conformation might be energetically more stable than an anchorage in the membrane. Some micro-micelles might contain more than one tetramer, in agreement with the demonstration in gel-filtration chromatography of larger structures besides the main fraction of M_r 310 000. One alternative hypothesis to explain the existence of a soluble form of the ecto-5'-nucleotidase would be that mechanical forces during homogenization of the tissue could extract from the membrane tetramers still containing four intact anchors. This is unlikely, since 15 strokes of the pestle yielded only 50% more soluble activity, compared with one stroke.

On the basis of the present data, it cannot be decided whether the transformation of the membrane-bound ecto-5'-nucleotidase into the soluble 'low- K_m ' 5'-nucleotidase occurs *in vitro* or *in vivo*. In the first case, the solubilization would be clearly of no physiological relevance. In the second case, the soluble enzyme would occur in the extracellular medium, and not in the cytosol,

in contrast with former expectations. The 'high- K_m ' ATP-activated soluble 5'-nucleotidase is therefore likely to represent the only enzyme capable of hydrolysing 5'-mononucleotides in the cytosol of the rat kidney (Bontemps *et al.*, 1989).

REFERENCES

- Biber, J., Stieger, B., Haase, W. & Murer, H. (1981) *Biochim. Biophys. Acta* **647**, 169–176
- Bontemps, F., Vincent, M. F., Van den Bergh, F., Vanwaeg, G. & Van den Berghe, G. (1989) *Biochim. Biophys. Acta* **997**, 131–134
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Dawson, T. P., Ghandi, R., Le Hir, M. & Kaissling, B. (1989) *J. Histochem. Cytochem.* **37**, 39–47
- Dieckhoff, J., Knebel, H., Heidemann, M. & Mannherz, H. G. (1985) *Eur. J. Biochem.* **151**, 377–383
- Fini, C., Camici, M., Minelli, A., Floridi, A. & Ipata, P. L. (1986) *Int. J. Biochem.* **18**, 683–689
- Fox, J. A., Soliz, N. M. & Saltiel, A. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2663–2667
- Fredholm, B. B. & Lindgren, E. (1983) *Biochem. Pharmacol.* **32**, 2832–2834
- Fritzon, P., Haugen, T. B. & Tiernshaugen, H. (1986) *Biochem. J.* **239**, 185–190
- Harb, J., Meflah, K., Duflos, Y. & Bernard, S. (1983) *Eur. J. Biochem.* **137**, 131–138
- Hawrylak, K. & Stinson, R. A. (1988) *J. Biol. Chem.* **263**, 14368–14373
- Headrick, J. P. & Willis, R. J. (1989) *Biochem. J.* **261**, 541–550
- Hjelmeland, L. M. & Chrambach, A. (1984) *Methods Enzymol.* **104**, 305–318
- Imai, S., Chin, W. P., Jin, H. & Nakazawa, M. (1989) *Pfluegers Arch.* **414**, 443–449
- Itoh, R. (1981) *Biochim. Biophys. Acta* **657**, 402–410
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Le Hir, M. & Dubach, U. C. (1988) *Am. J. Physiol.* **254**, F191–F195
- Le Hir, M., Gandhi, R. & Dubach, U. C. (1989) *Enzyme* **41**, 87–93
- Low, M. G. (1989) *Biochim. Biophys. Acta* **988**, 427–454
- Low, M. G. & Prasad, A. R. S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 980–984
- Madrid-Marina, V. & Fox, I. H. (1986) *J. Biol. Chem.* **261**, 444–448
- Mallol, J. & Bozal, J. (1983) *J. Neurochem.* **40**, 1205–1211
- Meghji, P., Middleton, K. M. & Newby, A. C. (1988) *Biochem. J.* **249**, 695–703
- Montero, J. M. & Fes, J. B. (1982) *J. Neurochem.* **39**, 982–989
- Naito, Y. & Lowenstein, J. M. (1985) *Biochem. J.* **226**, 645–661
- Newby, A. C. (1984) *Trends Biochem. Sci.* **9**, 42–44
- Newby, A. C. & Holmquist, C. A. (1981) *Biochem. J.* **200**, 399–403
- Panagia, V., Heyliger, C. E., Singal, P. K. & Dhalla, N. S. (1986) *J. Mol. Cell. Cardiol.* **18**, 815–826
- Ramos-Salazar, A. & Baines, A. D. (1986) *J. Pharmacol. Exp. Ther.* **263**, 494–499
- Ribeiro, J. A. & Sebastiao, A. M. (1987) *J. Physiol. (London)* **384**, 571–585
- Schütz, W., Schrader, J. & Gerlach, E. (1981) *Am. J. Physiol.* **240**, H963–H970
- Shryok, J. C., Rubio, R. & Berne, R. M. (1988) *Am. J. Physiol.* **254**, H223–H229
- Slavik, M., Kartner, N. & Riordan, J. C. (1977) *Biochem. Biophys. Res. Commun.* **75**, 342–349
- Spychala, J., Madrid-Marina, V., Nowak, P. J. & Fox, I. H. (1989) *Am. J. Physiol.* **256**, E386–E391
- Thompson, L. F., Ruedi, J. M. & Low, M. G. (1987) *Biochem. Biophys. Res. Commun.* **145**, 118–125
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Van Belle, H., Goossens, F. & Wynants, J. (1987) *Am. J. Physiol.* **252**, H886–H893
- Van den Berghe, G., Van Pottelsberghe, C. & Hers, H.-G. (1977) *Biochem. J.* **162**, 611–616

Received 18 June 1990/3 September 1990; accepted 24 September 1990