Adenosine increases blood flow and decreases excitatory nerve firing. In the heart, it reduces rate and force of contraction and preconditions the heart against injury by prolonged ischemia. Based on indirect kinetic arguments, an AMP-selective cytosolic 5′-nucleotidase designated cN-I has been implicated in adenosine formation during ATP breakdown. The molecular identity of cN-I is unknown, although an IMP/GMP-selective cytosolic 5′-nucleotidase (cN-II) and an ecto-5′-nucleotidase (e-N) have been cloned. We utilized the high abundance of cN-I in pigeon heart to purify a 40-kDa subunit for partial protein sequencing and subsequent cDNA cloning. We obtained a full-length clone encoding a novel 40-kDa peptide, unrelated to cN-II or e-N, that was most abundant in heart, brain, and breast muscle. Immunolocalization in heart showed a striated cytoplasmic location, suggesting association with contractile elements. Transient expression in COS-7 cells, generated a 5′-nucleotidase that catalyzed adenosine formation from AMP, which was increased during ATP catabolism. In conclusion, the cloning and expression of cN-I provides definitive evidence of its ability to produce adenosine during ATP breakdown.

The formation of adenosine during net catabolism of cytosolic ATP has been proposed to function as a signal of metabolic stress that brings about appropriate retaliatory actions against the conditions leading to stress (1, 2). The actions of adenosine include decreased stimulatory neurone firing and hyperpolarization of postsynaptic cells and antagonism of the effects of catecholamines on adenylate cyclase (3, 4). A3 receptor-mediated effects include vasodilatation leading to the increased delivery of substrates and oxygen to underperfused tissues (4, 5). In the heart, these mechanisms lead to a spectrum of responses including decreased heart rate, slower atrioventricular conduction, decreased force of contraction, and increased blood flow, all of which tend to reverse an imbalance between ATP formation and utilization (3, 6). Adenosine, acting through A1 or A3 receptors, also appears to play a role in ischemic preconditioning, a process by which brief ischemia can protect the heart against a subsequent longer period of infarction (4, 7, 8).

Cloning of e-N1 (EC 3.1.3.5) (9) confirmed a wealth of biochemical findings that this enzyme is involved exclusively in the hydrolysis of extracellular AMP, produced for example during cholinergic nerve transmission (10) and at the endothelial surface from degranulating platelets (11). Despite their importance, the mechanisms responsible for cytosolic AMP breakdown to adenosine have been elusive until now solely on indirect kinetic arguments. These have led to the conclusion that an IMP-preferring cytosolic 5′-nucleotidase, CN-II, abundant in the liver, probably plays a minor role in adenosine formation, especially in tissues such as heart that have an active AMP deaminase (12). An AMP preferring enzyme, cN-I, has been purified from pigeon, rabbit, rat, dog, and human hearts (13–17). The kinetic properties of cN-I, particularly its potent activation by ADP but not ATP (14, 15) and regulation by pH (18), are more consistent with a role in adenosine formation. However, the high apparent Ka for the enzyme of 5 mM (obtained at the optimum pH and with ADP concentrations above 50 µM) has cast doubt on its ability to generate adenosine under the conditions of the cell. Indeed, the capacity of any cytosolic 5′-nucleotidase to generate adenosine in intact cells has not previously been tested directly. The primary purpose of the present experiments was therefore to clone cN-I. Furthermore, we sought to obtain the first definitive evidence that a cytosolic 5′-nucleotidase was capable of catabolizing cytosolic AMP to adenosine under the conditions present within cells, particularly after metabolic poisoning to induce ATP breakdown.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Sequencing** —The cN-I enzyme was purified from pigeon (Columba livia) heart ventricles as described (14). A 40-kDa peptide was excised from 10% SDS-polyacrylamide gels, and peptides were generated by in situ tryptic proteolysis, separated by small bore reverse phase hplc, and N-terminally sequenced by Edman degradation (19).

**Cloning of the cDNA of cN-I** —A pigeon ventricle directional cDNA library was constructed in the vector λZap II (Stratagene, Cambridge, UK). RNA was extracted from one pigeon heart apex using ULTRA-SPEC (AMS, Witney, UK), poly(A)+ RNA was isolated using Oligotex kit (Qiagen, Crawley, UK). Degenerate antisense oligonucleotide primers were designed from the microsequenced peptide doubly underlined in Fig. 1. This was used paired with an oligonucleotide primer corresponding to the T3 promoter sequence present at the 5′ end of the λ phage cloning site. Touchdown PCR (20) was performed on 1 µl of the amplified pigeon cDNA library (corresponding to 3 × 108 plaque forming units or 20 copies of each clone). The PCR product was subcloned into pGEM-T (Promega, Chilworth, UK), and a clone coding for the remainder of the peptide sequence was obtained. This fragment (corresponding to nucleotides 107–285; Fig. 1) was excised, 32P-labeled by random priming, and used to screen 5 × 105 plaques from the library using the Rapid-hyb system (Amersham Pharmacia Biotech, Little Chalfont, UK). Pure cN-I from positive clones was in vivo excised to generate clones in the pBluescript SK+ plasmid as described by the manufacturer. Automated sequencing was conducted in both directions using the 5′-nucleotidease; hplc, high pressure liquid chromatography; PCR, polymerase chain reaction; LDH, lactate dehydrogenase.

---

* This work was supported by grants from the British Heart Foundation and the British Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s)M131243.

‡ To whom correspondence should be addressed. Tel.: 44-1179283552; Fax: 44-1179283538; E-mail: A.Newby@bris.ac.uk.

§ The abbreviations used are: e-N, ecto-5′-nucleotidase; CN, cytosolic 5′-nucleotidase; hplc, high pressure liquid chromatography; PCR, polymerase chain reaction; LDH, lactate dehydrogenase.
dideoxy chain termination method and an ABI (Perkin-Elmer, Applied Biosystems Division, Warrington, UK) automated sequencer.

Expression of the cN-I cDNA in COS-7 Cells—The full-length cDNA clone was subcloned into the BamHI-KpnI site of pTarget (Promega). COS-7 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin. COS-7 cell were transfected in 6-well plates using per well 10 μl of LipofectAMINE (Life Technologies, Inc., Paisley, UK) with 0.8 μg of cN-I coding plasmid plus 0.2 μg of pSV-β-galactosidase (Promega) control plasmid or 1 μg of pSV-β-galactosidase control plasmid and used 48 h after transfection. Cells to be transfected in KRH containing 120 μM NaCl, 48 μM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.3 mM CaCl2, 25 mM HEPES, pH 7.3) and preincubated for 15 min with 10 μM ethylnol-9-(2-hydroxy-3-nonyl)adenine, an inhibitor of adenosine deaminase (EC 3.5.4.4) (21). Cells were then incubated for 5 min in fresh medium containing ethylnol-9-(2-hydroxy-3-nonyl)adenine and 50 μM 5′-aminodeoxyadenosine to inhibit adenosine kinase (EC 2.7.1.20) (21). Glycosylation was inhibited by addition of 10 μM 2-deoxy-i-glucose, and mitochondrial ATP synth was inhibited with 1 μM carbonyl cyanide p-trifluromethoxy)phenyl-hydrazone. Cell incubations were terminated and metabolite and protein concentrations were determined as described below. The cell viability was assessed by measurement of LDH in cellular supernatants at the end of the incubations (cytotoxicity test, Roche Molecular Biochemicals, Lewis, UK) and expressed as the percentage of the total LDH present in parallel wells extracted in KRH containing 0.1% Triton X-100.

Characterization of Recombinant cN-I in COS-7 Cell Extracts—Untransfected and cN-I-transfected COS-7 cells were extracted with a buffer containing 40 mM N-dimethylglutarate, 40 mM β-glycerolphosphate, 200 mM KCl, 0.2 mM dithiothreitol, 12.6 mM MgCl2, 0.2% Triton X-100, pH 6.9. Extracts were subjected to polytronic homogenization for 15 s and then centrifuged at 10,000 × g for 10 min at 4 °C. Aliquots of extract were incubated for between 0 and 10 min at 37 °C with 10 μM ethylnol-9-(2-hydroxy-3-nonyl)adenine, 10 μM 5′-deoxy-5′-aminoadenosine, 50 μM α,β-methylene-ADP (final concentrations) and with 4.8 mM ATP, 2.8 mM ADP, 0.66 mM AMP, or (for the Kcat determinations) a range of AMP concentrations as described previously (22). Adenosine and protein concentrations were measured as described below.

Measurement of Nucleoside, Nucleotide, and Protein Concentrations—Addition of 0.75 volumes of 1.6 M HClO4 was used to terminate reactions followed by neutralization of supernatants with 0.125 volumes of 3 M K2PO4. Nucleosides and nucleotides were measured by reverse phase hplc, and transferred to Hybond N+ nylon membrane and hybridized using Rapid-hyb (Amersham Pharmacia Biotech) following the manufacturer's instructions.

Cloning of Cytosolic 5′-Nucleotidase

A rabbit polyclonal anti-cN-I antibody was generated by immunization with a multiple antigenic peptide (cytotoxicity test, Roche Molecular Biochemicals, Lewis, UK) and expressed as the percentage of the total LDH present in parallel wells extracted in KRH containing 0.1% Triton X-100.

Characterization of Recombinant cN-I in COS-7 Cell Extracts—Untransfected and cN-I-transfected COS-7 cells were extracted with a buffer containing 40 mM N-dimethylglutarate, 40 mM β-glycerolphosphate, 200 mM KCl, 0.2 mM dithiothreitol, 12.6 mM MgCl2, 0.2% Triton X-100, pH 6.9. Extracts were subjected to polytronic homogenization for 15 s and then centrifuged at 10,000 × g for 10 min at 4 °C. Aliquots of extract were incubated for between 0 and 10 min at 37 °C with 10 μM ethylnol-9-(2-hydroxy-3-nonyl)adenine, 10 μM 5′-deoxy-5′-aminoadenosine, 50 μM α,β-methylene-ADP (final concentrations) and with 4.8 mM ATP, 2.8 mM ADP, 0.66 mM AMP, or (for the Kcat determinations) a range of AMP concentrations as described previously (22). Adenosine and protein concentrations were measured as described below.

Measurement of Nucleoside, Nucleotide, and Protein Concentrations—Addition of 0.75 volumes of 1.6 M HClO4 was used to terminate reactions followed by neutralization of supernatants with 0.125 volumes of 3 M K2PO4. Nucleosides and nucleotides were measured by reverse phase hplc, and transferred to Hybond N+ nylon membrane and hybridized using Rapid-hyb (Amersham Pharmacia Biotech) following the manufacturer's instructions.

Immunochemistry and Western Blots—A rabbit polyclonal antibody was generated by immunization with a multiple antigenic peptide (25) of amino acids 222–233. The specificity of the antibody was confirmed by Western blot analysis. Equal quantities of protein (35 μg/lane) extracted from pigeon ventricle, untransfected COS-7 cells, and COS-7 cells expressing cN-I were fractionated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose. Antibody binding was detected by ECL (Amersham Pharmacia Biotech). Immunochemistry was conducted using the peroxidase antiperoxidase method (26) on 4-μm frozen sections of pigeon ventricle that had been fixed with cold aceton.

Statistical Methods—Values are expressed throughout as the means ± S.E. Comparisons are made by the Student's t test using paired data.

RESULTS

We took advantage of the high abundance of cN-I in pigeon heart to purify the enzyme using a published method (14). The product yielded a major 40-kDa subunit on SDS-polyacrylamide gel electrophoresis (results not shown). The band was excised and digested with trypsin, peptides were separated by hplc, and four partial N-terminal sequences were obtained (19–21) (underlined in Fig. 1). One sequence (doubly underlined in Fig. 1) was used to design degenerate oligonucleotide primers for PCR. The predicted peptide sequence used to generate a polyclonal antibody is in bold.

**FIG. 1.** Nucleotide and deduced amino acid sequence of cN-I cDNA. The asterisks indicate stop codons. The amino acid sequences determined are underlined. The doubly underlined sequence was used to design degenerate oligonucleotide primers for PCR. The predicted peptide sequence used to generate a polyclonal antibody is in bold.
e-N (9, 29). We therefore reached the surprising conclusion that cN-I is a novel protein, unrelated to cN-II or e-N. The clone did, however, contain a sequence (amino acids 253–336) with 79% identity to a translated human testis expressed sequence tag (accession number AA446194), implying this EST might code for a human cN-I.

The cDNA subcloned into pTargetT for expression in COS-7 cells directed expression of 5′-nucleotidase activity measured under conditions previously shown to be optimal for the detection of pigeon heart cN-I (22). Activities were 0.23 ± 0.02 μmol min⁻¹ mg protein⁻¹, which compared with activities of 0.004 ± 0.001 in untransfected cells. Parallel experiments using a plasmid encoding active β-galactosidase indicated that more than 30% of COS-7 cells were transfected under these conditions. The activity in transfected COS-7 cells was approximately 14-fold that previously reported in pigeon heart (14). The 5′-nucleotidase activity had properties similar to published values from pigeon heart (Table I). In particular, the cloned enzyme had a Km (measured at pH 7.0 and with greater than 50 μM ADP) similar to that of the pigeon heart cN-I and had a similar absolute requirement for adenine nucleotides as an activator. The enzyme had a preference for AMP over IMP and was only weakly inhibited by isobutylthioadenosine, which distinguishes it from cN-II. Like purified cN-I, it was inhibited by p-nitrophenol and also by 2′,3′-dideoxyctydine, dideoxymethidine, and dideoxyuridine, which have recently been shown to be potent and selective inhibitors of cN-I but not cN-II from rabbit heart (30).

Incubation of cells with inhibitors of adenosine deaminase and adenosine kinase was used to measure rates of adenosine formation from intact COS-7 cells (Fig. 2). Incubation with 2-deoxy-d-glucose and carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone was used to accelerate ATP catabolism. The effects of cN-I and β-galactosidase transfection (as a control for the effects of transfection per se) were compared with untransfected cells. In untransfected cells, the sum of ATP + ADP + AMP + adenosine was 107 ± 9 nmol/mg protein (n = 6). Purine metabolite concentrations remained unchanged during a control incubation for 5 min in the presence of inhibitors of adenosine metabolism (Fig. 2). Metabolic poisoning led to a 91 ± 1% fall in ATP concentration in 5 min, 85 ± 3% of which was accounted for by increased AMP concentration and 4 ± 1% of which was accounted for by increased adenosine concentration.

IMP inosine and hypoxanthine concentrations remained undetectable (data not shown). These results indicate that flux through endogenous 5′-nucleotidase and AMP deaminase was low under these conditions. Cells transfected with the control β-galactosidase encoding plasmid had a similar initial total adenine metabolite (ATP + ADP + AMP + adenosine) concentration (103 ± 20 nmol/mg, n = 4). However, in contrast to untransfected cells, they showed a significant fall in ATP concentration (p < 0.05) and rise in AMP concentration (p < 0.01) over 5 min of control incubation (Fig. 2). This adverse effect of transfection did not result in any increase in adenine concentration (Fig. 2). After incubation with metabolic poison ATP concentration fell to levels similar to those in untransfected cells. Of the fall in ATP concentration, 99 ± 2% was accounted for by increased AMP concentration and 4 ± 1% was accounted for by increased adenosine concentration (Fig. 2), similar to values in untransfected cells. Cells transfected with the cN-I plasmid had a significantly lower initial total adenine metabolite concentration (64 ± 7 nmol/mg, n = 6) compared with untransfected cells (p < 0.05). However, when values were
normalized (Fig. 2), they showed the same percentage fall in ATP concentrations during incubation without metabolic poisons as the cells transfected with β-galactosidase. In contrast to the β-galactosidase-transfected cells, there was no increase in AMP concentrations, but instead adenosine concentrations rose significantly (p, 0.02). These data show that there was basal flux through cN-I under these conditions. After metabolic poisoning of cN-I-transfected cells, ATP concentrations fell to a similar extent as in untransfected or β-galactosidase-transfected cells. However, only 60 ± 6% of the fall in ATP concentration was accounted for by increased AMP concentration, whereas 39 ± 3% was accounted for by increased adenosine concentration (both p, 0.001 versus either untransfected or β-galactosidase-expressing control cells). From these data it is clear that AMP was stoichiometrically converted to adenosine in cN-I-transfected cells. Metabolic poisons clearly increased adenosine formation by 2.6-fold (Fig. 2).

To control for possible effects on cell viability, we measured release of LDH under the various experimental conditions. Untransfected cells released 0.3 ± 0.2 and 1.3 ± 0.7% of LDH during incubation without and with metabolic poisons (n = 4). Cells transfected with β-galactosidase or cN-I released similarly increased levels of LDH (1.5 ± 1.3 and 2.6 ± 1.6%, respectively, without and 2.5 ± 1.7 and 4.6 ± 1.6%, respectively, with metabolic poisons). These increases in LDH release are consistent with the evidence from measurements of ATP and AMP concentrations that transfection per se causes metabolic stress. However, the effects on LDH release were small compared with the rises in adenosine in cN-I-expressing cells, which implies that most adenosine formation occurred inside intact cells. Further data were obtained against the possibility that released AMP metabolized by e-N accounted for the rises in adenosine formation. The selective e-N inhibitor, α,β-methylene-ADP (50 μM), did not decrease the percentage of ATP converted to adenosine in two separate batches of metabolically poisoned, cN-I-expressing cells (28 or 56% without α,β-methylene-ADP versus 41 or 61% with α,β-methylene-ADP).

The distribution of cN-I was investigated by reverse transcriptase-PCR (not shown) and Northern blotting (Fig. 3). Reverse transcriptase-PCR products of the correct size were obtained from all the pigeon tissues tested, albeit at trace levels in aorta, liver, and kidney. Products were most abundant in heart, brain, and skeletal (breast) muscle. These results were corroborated by Northern analysis (Fig. 3), in which a single 2.3-kilobase transcript was prominent in heart, brain, and breast muscle.

The distribution of cN-I in pigeon ventricle was further investigated by immunocytochemistry using a polyclonal antibody raised against the peptide indicated in Fig. 1. The antibody recognized a predominant 40-kDa band in extracts of pigeon heart and in cN-I-transfected but not untransfected COS-7 cells (Fig. 4a). Staining of frozen sections showed diffuse cytoplasmic staining of myocytes (Fig. 4b, c and d), which gave a striated pattern in cells sectioned longitudinally (Fig. 4, b and c).

**DISCUSSION**

An IMP-selective cytosolic 5′-nucleotidase (cN-II) (27, 28) and an ecto-5′-nucleotidase (e-N) (9) have been previously cloned. One of the most surprising conclusions of our study is that the sequence of cN-I bears no similarity to cN-II. Interestingly e-N is related neither to cN-I nor to cN-II. Presumably the potent, selective activation of cN-I, the less potent and less selective activation of cN-II, and inhibition of e-N by ADP represent cases of convergent evolution. The only recognizable

**FIG. 4. Location of cN-I in pigeon heart by immunocytochemistry.** a, the specificity of the rabbit polyclonal antibody anti cN-I was confirmed by Western blot analysis. Equal quantities of protein (35 μg/lane) extracted from pigeon ventricle, untransfected COS-7 cells, and COS-7 cells expressing cN-I were fractionated by SDS-polyacrylamide gel electrophoresis (10%). b–d, immunoperoxidase staining (26) of 4-μm frozen sections of pigeon heart fixed with acetone using a 1:400 dilution of antiserum against cN-I (b and c) or preimmune rabbit serum (d). The counterstain is hematoxylin. Scale bars correspond to 25 μm.

**Cloning of Cytosolic 5′-Nucleotidase**
structural motif in cN-I was a putative nuclear localization signal, and this proved to be inactive, as indicated by immunocytochemistry. Indeed, cN-I was confirmed to be cytoplasmic, in agreement with a previous study (31). However, our results imply association with the contractile apparatus, as has been suggested previously from subcellular fractionation studies. These experiments (22) showed that the addition of KCl to buffers used for homogenization led to a redistribution of cN-I from the particulate to the soluble fraction, implying a loose interaction with contractile elements. This association may provide a mechanism for sensing local AMP concentrations in the region of the contractile proteins. Our results showed that cN-I is abundant in oxidative muscles (heart and breast muscle) and also in brain.

Adenosine production from endogenous pathways amounted to 4% of ATP breakdown in untransfected cells. We therefore had to obtain expression of recombinant cN-I in a substantial proportion of cells to exceed this background. We achieved this by liposome-mediated transfection and used transfection with the irrelevant gene, β-galactosidase, as a control. Our results demonstrate that transfection per se caused metabolic stress, as evidenced by increased AMP concentrations under basal conditions. Given this, it was not surprising that when we inhibited adenosine metabolism, we demonstrated substantial adenosine formation in cN-I-transfected cells, even in the absence of metabolic poisons. However, flux through the enzyme was clearly elevated during ATP catabolism, consistent with the role proposed for cN-I in catalyzing the cytoprotective actions of adenosine. Interestingly, overexpression of cN-I also caused a long term decline in total adenine nucleotide concentrations in the absence of inhibitors of adenosine metabolism. It will be interesting to investigate in future experiments whether decreasing cN-I activity causes a corresponding increase in the total adenine nucleotide pool.

In conclusion, the aims of this study were to clone cN-I and provide definite evidence for its role in ATP catabolism and adenosine formation. Our results clearly demonstrate that we have achieved these two aims.

Acknowledgments—We thank Kate Keen and Jason Johnson for the expert technical assistance. Protein sequence analysis was performed by Dr. M. Wilkinson (University of Liverpool, Liverpool, UK).

REFERENCES
1. Newby, A. C. (1984) Trends Biochem. Sci. 9, 42–44
2. Newby, A. C., Worku, Y., Meghji, P., Nakazawa, M., and Skladanowski, A. C. (1990) Neur Physiol. Sci. 5, 67–70
3. Schrader, J. (1990) Circulation 81, 389–391
4. Ralevic, V., and Burnstock, G. (1998) Pharmacol. Rev. 50, 413–492
5. Olsson, R. A., and Pearson, J. D. (1990) Physiol. Rev. 70, S61–S85
6. Berne, R. M. (1984) Physiol. Rev. 64, 1–29
7. Yokota, R., Fujwara, H., Miyamase, M., Tanaka, M., Yamashita, K., Itoh, S., Koga, K., Yabuuchi, Y., and Sasayama, S. (1995) Am. J. Physiol. 268, H1149–H1157
8. Cohen, M. V., Walsh, R. S., Goto, M., and Downey, J. M. (1995) J. Mol. Cell. Cardiol. 27, 1527–1534
9. Misumi, Y., Ogata, S., Hirose, S., and Ikeharoko, Y. (1990) J. Biol. Chem. 265, 2178–2183
10. Richardson, P. J., Brown, S. J., Bailey, E. M., and Luzio, J. P. (1987) Nature 327, 232–234
11. Gordon, E. L., Pearson, J. D., and Slakey, L. L. (1986) J. Biol. Chem. 261, 15486–15507
12. Skladanowski, A. C., and Newby, A. C. (1991) in Role of Adenosine and Adenine Nucleotides in the Biological System (Imai, S., and Nakazawa, M., eds) pp. 289–300, Elsevier Science, Amsterdam
13. Truong, V. L., Collinson, A. R., and Lowenstein, J. M. (1988) Biochem. J. 253, 117–121
14. Skladanowski, A. C., and Newby, A. C. (1990) Biochem. J. 268, 117–122
15. Yamazaki, Y., Truong, V. L., and Lowenstein, J. M. (1991) Biochemistry 30, 1503–1509
16. Skladanowski, A. C., Smolenski, R. T., Tavernier, M., de Jong, J. W., Yacoub, M. H., and Seymour, A. M. L. (1996) Am. J. Physiol. 270, H1493–H1500
17. Darvish, A., and Metting, P. J. (1993) Circulation 98, 117–121
18. Darvish, A., Matteo, R., Metting, P., Stewart, R. W., and Moravee, C. S. (1996) Circulation 94, 722–729
19. Rosenfeld, J., Capdeville, J., Gillemot, J. C., and Ferrara, P. (1992) Anal. Biochem. 203, 173–179
20. Hecker, K. H., and Roux, K. H. (1996) BioTechniques 21, 478–485
21. Newby, A. C. (1981) Biochem. Pharmacol. 30, 2611–2615
22. Newby, A. C. (1988) Biochem. J. 253, 123–130
23. Skladanowski, A. C., Smolenski, R. T., Ledingham, S. J. M., and Yacoub, M. H. (1990) J. Chromatogr. 527, 414–420
24. Davis, L. G., Bibner, M. D., and Battey, J. F. (1995) in Basic Methods in Molecular Biology, pp. 143–146, Appleton and Lange, Norwalk.
25. Posnett, D. N., and Tam, J. P. (1989) Methods Enzymol. 178, 739–746
26. Sternberger, L. A. (1979) J. Histochem. Cytochem. 27, 1657
27. Oka, J., Matsumoto, A., Husokawa, Y., and Inoue, S. (1995) Biochem. Biophys. Res. Commun. 205, 917–922
28. Allegrini, S., Pesi, R., Tsui, M. G., Fiol, C. J., Johnson, R. B., and Eriksson, S. (1997) Biochem. J. 328, 483–487
29. Suzuki, K., Furukawa, Y., Tanura, H., Ejiri, N., Suehata, H., Taguchi, R., Nakamura, S., Suzuki, Y., and Ikezawa, H. (1998) J. Biochem. 113, 507–513
30. Garvey, E. P., Lowen, G. T., and Almond, M. R. (1998) Biochemistry 37, 9043–9051
31. Darvish, A., Pastewaite, J. J., and Metting, P. J. (1993) Hypertension 21, 906–910