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Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide

(cardiovascular control/blood pressure/synthetic peptide/DNA cloning)

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ABSTRACT **Endothelin is a potent vasoconstrictor/pressor peptide, which we recently characterized from the conditioned culture medium of porcine aortic endothelial cells. We report here the cloning and partial sequencing of the rat endothelin gene. The nucleotide sequence predicted a 21-residue peptide similar to, but distinct from, porcine endothelin; 15 residues of rat endothelin were identical and 3 residues were substitutions by chemically similar amino acid residues to those in the porcine peptide. Synthetic rat endothelin was then prepared according to its deduced amino acid sequence. This synthetic peptide had (*i*) potent vasoconstrictor activity in the rat aortic strip and in perfused rat heart and (*ii*) a characteristically long-lasting *in vivo* pressor activity by intraaortic bolus injection in the conscious rat.**

Vascular tonus is regulated by various neural and hormonal stimuli together with regional regulatory mechanisms of the blood vessel wall, including the smooth muscle and endothelial layers. The discovery of acetylcholine-induced, endothelium-dependent vasodilatation by Furchgott (1) has stimulated intense interest in the role of the endothelium in modulating vascular responsiveness. Endothelin is an endothelial cell-derived vasoconstrictor/pressor peptide, which we originally isolated and sequenced from the culture medium of porcine aortic endothelial cells (2). Consisting of 21 amino acid residues with two sets of intrachain disulfide linkages, porcine endothelin is one of the most potent vasoconstrictors known. Sequence analysis of cloned porcine endothelin cDNA showed that porcine endothelin is produced in endothelial cells from a 203-residue prepropeptide much like many peptide hormones and neuropeptides. Further, we cloned a cDNA encoding human endothelin and showed that the amino acid sequence of human and porcine endothelin is identical (3). Porcine preproendothelin mRNA is expressed not only in the cultured endothelial cells but also in aortic endothelium *in vivo*, and the level of mRNA expression is markedly influenced by vasoactive agents such as thrombin and adrenaline (2). These observations suggest the existence in the mammalian cardiovascular system of a distinct endothelium-mediated regulatory mechanism for blood pressure and/or local blood distribution.

As a basis for investigation on the physiological and pathophysiological roles of this endogenous vasoconstrictor in rat, we have now cloned and partially sequenced the rat preproendothelin gene.‡ Further, rat endothelin was chemically synthesized according to the encoded amino acid sequence, and the synthetic peptide was shown to possess strong vasoconstrictor activities.

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MATERIALS AND METHODS

Cloning and Sequencing of Rat Endothelin Gene. A rat genomic library constructed in λCharon 4A was obtained from Clontech Laboratories (Palo Alto, CA). Approximately 10^6 plaques from the library were screened by hybridization with a nonredundant synthetic DNA probe encoding amino acid residues 7–20 of porcine endothelin as described (2). One hybridization-positive clone with a 12.3-kilobase (kb) insert, λgrET1, was identified and plaque purified. The rat endothelin coding sequence was located within a 1.4-kb *Pvu* II fragment by Southern blot analysis (4) with the same synthetic probe (Fig. 1). The relevant restriction fragments were subcloned in pUC118/119 plasmids, rescued as single-stranded DNA, and sequenced from both strands by the dideoxy nucleotide chain-termination method (5). Other standard recombinant DNA procedures were done as described (6).

Synthesis of Rat Endothelin. Rat endothelin was assembled by using an Applied Biosystems model 430A peptide synthesizer (software version 1.40) with phenylacetamidomethyl (PAM)-resin as the solid support. The final protected peptide resin was treated with anhydrous hydrogen fluoride in the presence of 5% (vol/vol) *p*-cresol/15% (vol/vol) butanedithiol at −2°C for 60 min. After evaporation of excess hydrogen fluoride, the crude peptide was precipitated with ether and extracted with CF_3COOH to filter off the solid support. The crude product containing four sulfhydryl groups was then subjected to air oxidation at a concentration of 0.1 mM in aqueous NH_4OH at room temperature for 16 hr and purified by preparative reverse-phase HPLC (Waters PrepLC System 500) with a 20–45% linear gradient of CH_3CN in 0.1% CF_3COOH. Overall yield from the peptide resin was 15%. Homogeneity of the final product was confirmed by analytical HPLC and amino acid analysis. The disulfide-bond topology of synthetic rat endothelin was determined as Cys-1—Cys-15 and Cys-3—Cys-11, which is identical to that of natural porcine endothelin, by the synthesis of the disulfide isomers with selective oxidation of the two pairs of cysteine residues. Details of the selective synthesis of the disulfide isomers will be described elsewhere.

Assay of *in Vitro* Vasoconstrictor Activities. Hearts and thoracic aortae were isolated from anesthetized male Wistar rats (300–400 g). Hearts were perfused basically according to Langendorff's method (12). After the right and left atria were removed, ventricles were perfused via aortic cannulae with Krebs–Ringer solution (113 mM NaCl/4.8 mM KCl/2.2 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/2.5 mM

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04075).

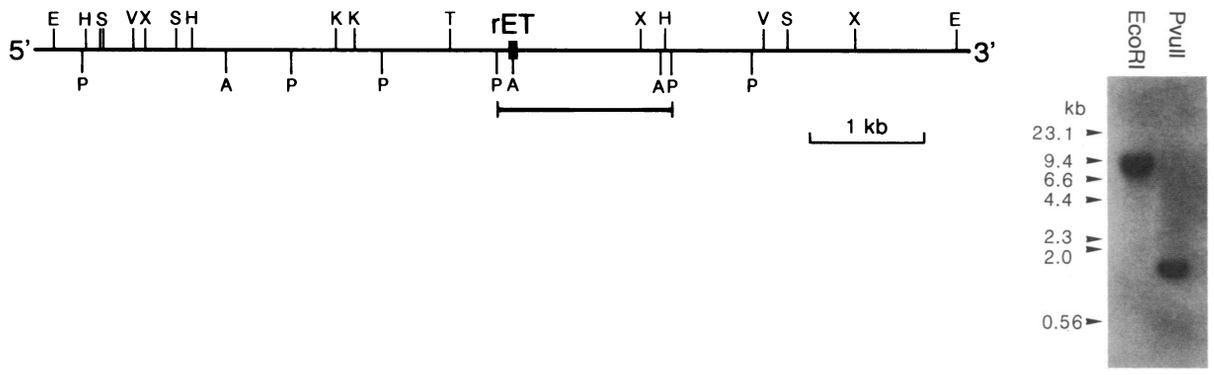

FIG. 1. (*Left*) Restriction map of rat preproendothelin gene cloned in λgrET1 insert. The sequence encoding mature rat endothelin (rET) is indicated by a closed box. A, *Acc* I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pvu* II; S, *Sst* I; T, *Pst* I; V, *Eco*RV; X, *Xba* I. Sites for *Acc* I and *Pvu* II are only partially characterized. (*Right*) Rat genomic DNA blot. The 1.4-kb *Pvu* II fragment of λgrET1 insert (indicated by a solid bar *Left*) was hybridized at 42°C in 50% (vol/vol) formamide. The membrane was washed in 0.3 M sodium chloride/30 mM sodium citrate at 65°C.

$NaHCO_3$/5.5 mM glucose) maintained at 37°C and aerated with 95% O_2/5% CO_2 at a constant flow rate of 5 ml/min. A pair of platinum electrodes were placed around the atrioventricular node, and the tissues were electrically driven at 3.3 Hz with square-wave pulses of 1-msec duration and 3-V intensity. Coronary perfusion pressure was measured from the perfusate line by pressure transducers. The aortae were cut into helical strips of 2 × 15 mm, and endothelium was removed by rubbing the surface with a swab. Both heart and smooth-muscle preparations were suspended in Krebs–Ringer solution maintained at 37°C and gassed with 95% O_2/5% CO_2, and the resting tensions applied were 3 g for the ventricles and 0.75 g for the aortae. The developed isometric tensions were recorded with force-displacement transducers (Nihon Koden, Tokyo).

Assay of *in Vivo* Pressor Effect in Conscious Rats. Male Sprague–Dawley rats (350–400 g) were used. The aortic blood pressure and the pulse rate were monitored without anesthesia or restraint (7) 2–10 days after a cannula was inserted into the abdominal aorta via the left femoral artery. Synthetic rat endothelin and angiotensin II were injected in bolus (1 ml/kg) through another aortic cannula inserted via the right femoral artery. The tip of this cannula was placed about 5 mm peripheral to the cannula for pressure measurement.

RESULTS

Primary Structure of Rat Endothelin. The partial restriction map of part of the rat endothelin gene contained in λgrET1 is shown (Fig. 1 *Left*). A genomic Southern blot was probed with the 1.4-kb *Pvu* II fragment of the λgrET1 insert, which contained the rat endothelin sequence (Fig. 1 *Right*). Single *Eco*RI (7.9 kb) and *Pvu* II (1.4 kb) fragments were detected, indicating that the insert of λgrET1 is an authentic copy of the genomic locus and that rat endothelin is encoded in a single-copy gene. A partial nucleotide sequence of the λgrET1 insert around the rat endothelin sequence is shown in Fig. 2. The sequence from nucleotides 34–75 matched well with the synthetic oligonucleotide probe used to screen the library (38/42 bases). The deduced amino acid sequence corresponding to the reading frame of the probe sequence is aligned in Fig. 2 with the corresponding portion of the porcine preproendothelin sequence. We concluded from the following that an intron starts from nucleotide 93: (*i*) the sequence here fits well with the consensus sequence for the 5′-end of the intron, GTRAGT where R = A or G; (*ii*) both the nucleotide and deduced amino acid sequences suddenly lose similarity to the porcine sequences beyond nucleotide 93; (*iii*) an exon/intron junction has been located at nucleotide 93 in the human endothelin gene (M.Y. and A.I., unpublished work).

Biological Activities of Synthetic Rat Endothelin. According to the primary structure predicted from the nucleotide sequence, we synthesized rat endothelin by solid-phase chemistry and assayed the biological activities of the synthetic peptide. The synthetic rat endothelin induced a slow-onset and long-lasting contraction of rat aortic strips (Fig. 3). The maximum tension increment was 160% of that for KCl-induced contraction, indicating that rat endothelin is an extremely strong constrictor for rat aortae. The estimated EC_{50} value in this assay was 5–6 × 10^{-8} M; this value is less

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r  CAC CGA CCT CGG CGC TGC ACG TGC TTC ACT TAT AAG GAC AAG GAG TGT GTC TAC TAC TGC   60
   His Arg Pro Arg Arg Cys Thr Cys Phe Thr Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys   20
p  Arg Arg Ser Lys Arg Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys   67
   CGT CGG TCC AAG CGC TGC TCC TGC TCT TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC  201

r  CAC CTG GAC ATC ATC TGG▼atc AAC ACT CCT GA gtgagttggcctggggttgggggaggagtgtggcct  128
   His Leu Asp Ile Ile Trp Ile Asn Thr Pro                                          30
p  His Leu Asp Ile Ile Trp Val Asn Thr Pro Glu His Ile Val Pro Tyr Gly Leu Gly Ser   87
   CAC CTG GAC ATC ATC TGG GTC AAC ACT CCA GAA CAC ATT GTC CCA TAC GGA CTT GGA AGC  261
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FIG. 2. Partial nucleotide and deduced amino acid sequences of rat preproendothelin gene aligned with porcine endothelin cDNA sequence (2). The nucleotide and amino acid residues that differ in rat (r) and porcine (p) sequences are underlined. The putative intron for the rat gene is in lowercase. Mature endothelin sequences are indicated by an open box. The unusual carboxyl-terminal processing site, which is presumably cleaved by an "endothelin-converting enzyme" is indicated by an arrow.

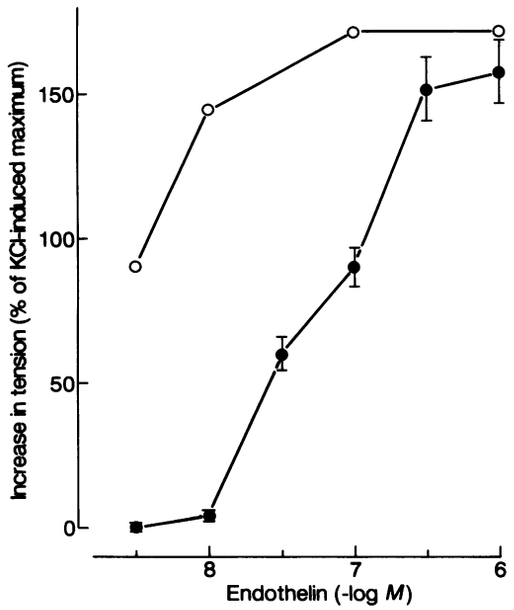

FIG. 3. Dose–response relationship for constrictive responses in deendothelialized rat aortic strips to cumulatively applied rat endothelin (●; $n = 6$) and porcine endothelin (○; $n = 2$).

potent by a factor of about 20 when compared with the EC_{50} of porcine endothelin assayed on rat aortic strips (3×10^{-9} M; Fig. 3), although it is still as potent as the classical peptide vasoconstrictor angiotensin II (8).

Rat endothelin also showed a strong coronary constrictor activity on perfused rat hearts *in vitro* (Fig. 4). When a 0.1- to 1.0-nmol bolus of rat endothelin was introduced via the aorta, coronary perfusion pressure increased in a dose-dependent manner. The maximum pressures were attained within 1 min after infusion of rat endothelin, and the pressure returned to the base-line levels after 10–30 min, depending on the dose of rat endothelin. Interestingly, the decay kinetics of this constrictor response was considerably different from that for porcine endothelin; the coronary pressure effect of porcine endothelin lasted for >1 hr in this assay system (T.I., unpublished work). Both rat endothelin and porcine endothelin showed a positive inotropic effect on isolated guinea pig atria (9), as anticipated from the proposal that endothelin acts through activation of the dihydropyridine-sensitive Ca^{2+} channels (2). However, in the Langendorff preparation shown in Fig. 4, rat endothelin exhibited very little inotropic effect. This result might be due to the lower sensitivity to endothelin of myocardium as compared with that of vascular smooth muscle.

In vivo, intraaortic bolus injection of rat endothelin exhibited a characteristically long-lasting pressor effect in conscious rats (Fig. 5). Rat endothelin (1 nmol/kg) caused a sustained rise of blood pressure lasting for ≈1 hr, which was preceded by a transient hypotensive response. The mechanism for the initial transient drop in blood pressure is at present unknown. A transient reactive tachycardia followed by a long-lasting suppression in heart rate accompanied these responses. The minimal effect dose of rat endothelin was 30 pmol/kg. Rat endothelin at 10 nmol/kg caused a 40-mmHg (1 mmHg = 1.333 × 10^2 Pa) rise in blood pressure, which approximately equals the maximum pressor effect of angiotensin II (100 pmol/kg). Fig. 5 also shows the pressor effect of angiotensin II as an example of response to a well-characterized pressor substance. In contrast to rat endothelin, angiotensin II exhibited a more potent, but very short-lasting, pressor activity. Blood pressure returned to base-line levels within 2–3 min. The minimum effective dose for angiotensin II was 3 pmol/kg, which was one order of magnitude less than that for rat endothelin.

DISCUSSION

The amino acid sequence of the portion of rat endothelin precursor shown in Fig. 2 is highly similar to, but distinct from, that of the porcine precursor. Of 30 amino acid residues in this segment of the rat precursor, 20 (67%) are identical and 26 (87%) are conservative substitutions (including identities) (10). Most significantly, the positions of the four cysteine residues, which might be an important determinant of the higher structure of the peptide, are perfectly conserved. Strong similarity is also seen at the nucleotide sequences (76%). The putative rat endothelin sequence is, as expected, directly preceded by a dibasic pair, Arg-4–Arg-5 (11). However, as for porcine (2) and human (3) precursors, no dibasic pair is found in the carboxyl-terminal region of the peptide. Although information at the peptide level is needed for the definite location of the carboxyl terminus, it is most likely that (from analogy to the porcine peptide) the carboxyl terminus of (mature) rat endothelin is Trp-26. Thus, we believe that rat endothelin is a 21-residue peptide of M_r 2643 with two intrachain disulfide bonds, which, like in porcine endothelin, is produced through unusual proteolytic processing at Trp-26–Ile-27 by an "endothelin-converting enzyme" (2).

Although rat endothelin is shown here to be a potent constrictor of rat blood vessels *in vitro* and an effective pressor agent *in vivo*, the details of the pharmacological

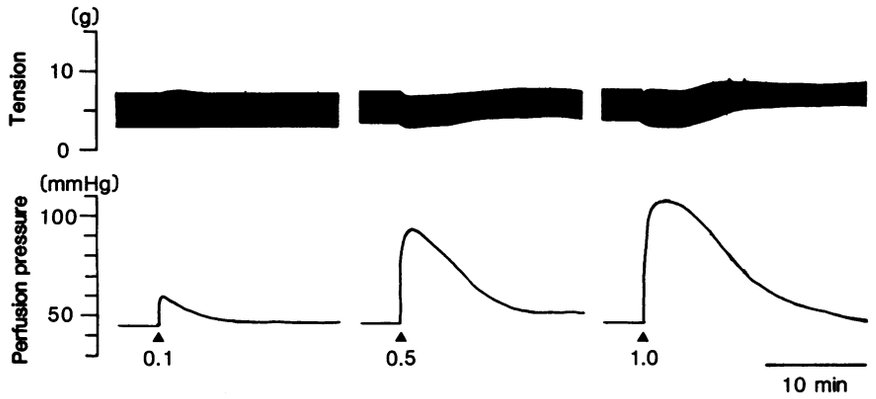

FIG. 4. Coronary constrictor effect of rat endothelin in a perfused rat heart. Increased doses (in nmol) of rat endothelin bolus were applied from the perfusate, and the ventricular contractile force (upper record) and coronary perfusion pressure (lower record) were measured.

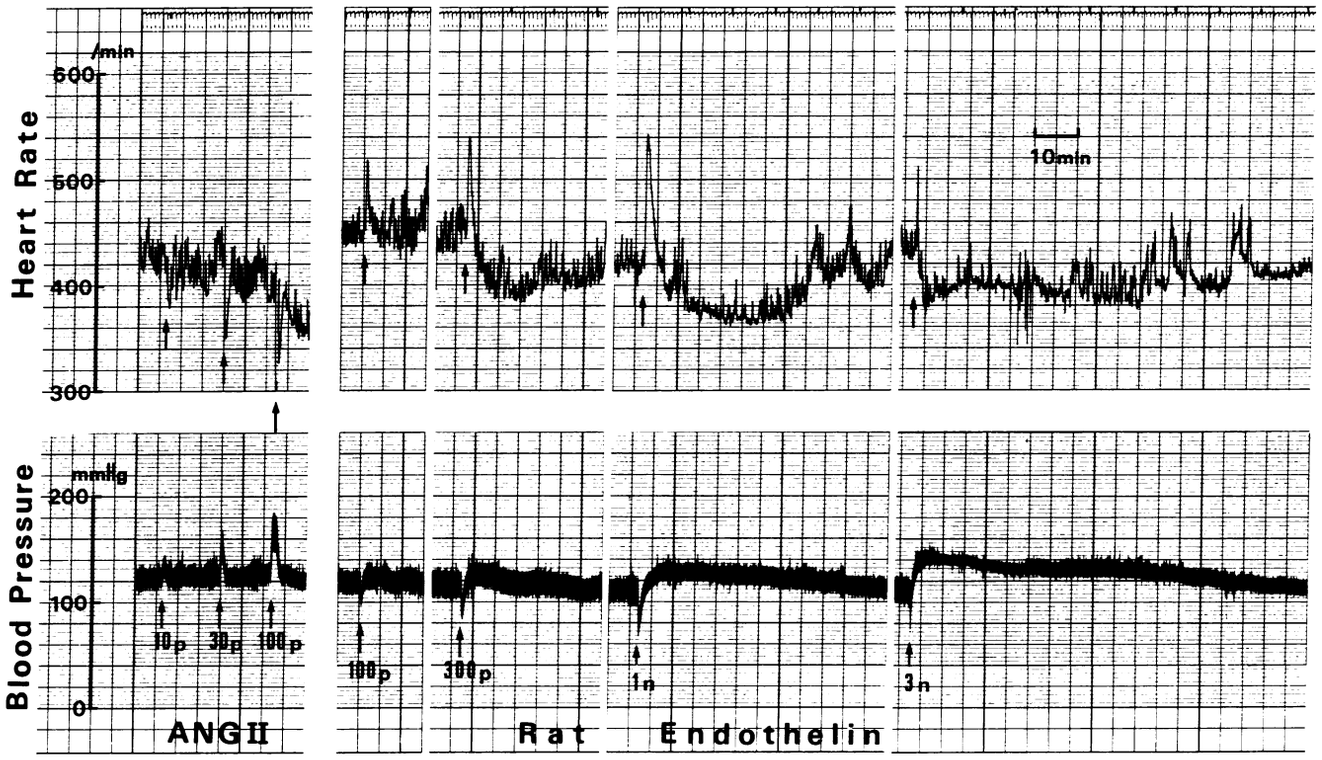

FIG. 5. *In vivo* pressor effect of angiotensin II (ANG II) and rat endothelin in a conscious rat. Increased doses (in mol/kg at arrows) of ANG II or rat endothelin were applied in a bolus from an intraaortic cannula, and the heart rate (upper record) and aortic pressure (lower record) were monitored.

activities are quite different between rat endothelin and porcine endothelin. Rat endothelin constricts rat aortic strips with less potency than does porcine endothelin, but perhaps more importantly, decay of the coronary pressor effect of rat endothelin was much more rapid than that of porcine endothelin. The amino acid sequence of rat endothelin and porcine endothelin differ chiefly in the amino-terminal halves of the peptides—rat endothelin being more polar because of the charged lysine residue instead of the hydrophobic methionine found in porcine endothelin. Rat endothelin could be more easily washed off the relevant receptors due to this more polar nature of the amino-terminal half of the molecule. In contrast, the carboxyl-terminal halves of rat endothelin and porcine endothelin are highly conserved; the carboxyl-terminal hexapeptide after the last half-cystine residue, His-Leu-Asp-Ile-Ile-Trp, is common in both endothelins. This carboxyl-terminal "tail" portion might be essential for the vasoconstrictor activity of endothelin.

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1. Furchgott, R. F. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24,** 175–197.
2. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) *Nature (London)* **332,** 411–415.
3. Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. & Masaki, T. (1988) *FEBS Lett.* **231,** 440–444.
4. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
5. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
6. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
7. Kawashima, K., Shiono, K. & Sokabe, H. (1980) *Clin. Exp. Hypertension* **2,** 229–245.
8. Regoli, D., Park, W. K. & Rioux, F. (1974) *Pharmacol. Rev.* **26,** 69–123.
9. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. & Masaki, T. (1988) *Am. J. Physiol.*, in press.
10. Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure*, ed. Dayhoff, M. O. (Natl. Biomed. Res. Found., Silver Spring, MD), Vol. 5, Suppl. 3, pp. 345–352.
11. Turner, A. J. (1986) *Essays Biochem.* **22,** 69–119.
12. Langendorff, D. (1985) *Pflügers Arch.* **61,** 291–332.