Identification and characterization of a tissue kallikrein in rat skeletal muscles

Nobuo SHIMOJO,*|| Julie CHAO,† Lee CHAO,‡ Harry S. MARGOLIUS*† and Ronald K. MAYFIELD*§¶

Departments of *Medicine, †Pharmacology and ‡Biochemistry, Medical University of South Carolina, 171 Ashley Avenue, and §Veterans Administration Medical Center, Charleston, SC 29425, U.S.A.

A tissue kallikrein was purified from rat skeletal muscle. Characterization of the enzyme showed that it has α -N-tosyl-L-arginine methylesterase activity and releases kinin from purified bovine low- M_r kininogen substrate. The pH optimum (9.0) of its esterase activity and the profile of inhibition by serine-proteinase inhibitors are identical with those of purified RUK (rat urinary kallikrein). Skeletal-muscle kallikrein also behaved identically with urinary kallikrein in a radioimmunoassay using a polyclonal anti-RUK antiserum. On Western-blot analysis, rat muscle kallikrein was recognized by affinity-purified monoclonal anti-kallikrein antibody at a position similar to that of RUK (M_r 38000). Immunoreactive-kallikrein levels were measured in skeletal muscles which have different fibre types. The soleus, a slow-contracting muscle with high mitochondrial oxidative-enzyme activity, had higher kallikrein content than did the extensor digitorum longus or gastrocnemius, both fast-contracting muscles with low oxidative-enzyme activity. Streptozotocin-induced diabetes reduced muscle weights, but did not alter the level of kallikrein (pg/mg of protein) in skeletal muscle, suggesting that insulin is not a regulator of kallikrein in this tissue. Although the role of kallikrein in skeletal muscle is unknown, its localization and activity in relation to muscle functions and disease can now be studied.

INTRODUCTION

Tissue kallikreins (EC 3.4.21.35) are serine proteinases that liberate kinin peptides from kininogen substrates (Miller & Margolius, 1986). Kinins are potent agonists *in vitro* of smooth-muscle contraction or relaxation (action is site-specific; Johnson, 1979) and epithelial ion transport (Cuthbert & Margolius, 1982; Leikauf *et al.*, 1985). Although it remains uncertain what, if any, physiological or pathological role kallikreins may play, recent studies have expanded the list of possible natural substrates. These include apolipoprotein B 100 (Cardin *et al.*, 1984), atriopeptigens (Currie *et al.*, 1984), β -nerve growth factor (Berger & Shooter, 1977), pro-renin (Sealy *et al.*, 1978) and pro-insulin (ole-Moi Yoi *et al.*, 1979). Many tissue sites not previously recognized as kallikrein-containing have been found to have a kallikrein that is enzymically and/or immunologically identical with the enzyme found in kidney, pancreas, intestinal mucosa and salivary glands. These new sites include brain (Chao *et al.*, 1983), pituitary (Powers & Nasjletti, 1983), vascular wall (Nolly *et al.*, 1985) and spleen (Chao *et al.*, 1984). Identification of kallikrein mRNA in brain (Chao *et al.*, 1983), pituitary (Chao *et al.*, 1987) and spleen (Swift *et al.*, 1982) constitutes proof that these tissues synthesize the enzyme. Together, all of these findings raise the possibility that members of the tissue-kallikrein family may serve broader functions than initially perceived.

Studies in isolated cells or tissues suggest that a tissue-kallikrein-like enzyme could be involved in the transmembrane signalling process which mediates some

actions of insulin (Jarett *et al.*, 1982; Lerner *et al.*, 1982; Seals & Czech, 1980). Furthermore, studies *in vivo* have shown that aprotinin, a kallikrein inhibitor, blocks the action of insulin on glucose and amino acid uptake in the human forearm, and that simultaneous bradykinin infusion restores the insulin effect (Dietze *et al.*, 1980). These reports prompted us to look for tissue kallikrein in skeletal muscle, the principal peripheral target organ for insulin. By using immunoaffinity chromatography with new monoclonal antibodies to rat tissue kallikrein (Woodley *et al.*, 1985), we have identified and characterized a tissue kallikrein from rat skeletal muscle. Levels of this kallikrein in individual muscles containing different fibre types were measured by radioimmunoassay in normal and STZ-diabetic rats.

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources: Tos-Arg-OMe, enzyme-grade (NH_4)₂SO₄ (Schwartz/Mann, Spring Valley, NY, U.S.A.); Tos-Arg-O[³H]Me (Amersham Corp., Arlington Heights, IL, U.S.A.); aprotinin, bovine serum albumin, SBTI, sodium deoxycholate, Sephadex G-25 (Sigma Chemical Co., St. Louis, MO, U.S.A.); LBTI (Worthington Biochemical Corp., Freehold, NJ, U.S.A.); polyacrylamide (Eastman Kodak Co., Rochester, NY, U.S.A.); especially pure SDS (BDH Chemicals, Poole, Dorset, U.K.); nitrocellulose paper BA 85, 0.45 μm pore size (Schleicher and

Abbreviations used: Tos-Arg-OMe, α -N-tosyl-L-arginine methyl ester; OMease, methylesterase; SBTI, soybean trypsin inhibitor; LBTI, lima-bean trypsin inhibitor; EDL, extensor digitorum longus; STZ, streptozotocin; RUK, rat urinary kallikrein.

|| Present address: Department of Laboratory Medicine and Central Clinical Laboratory, Osaka City University Medical School, Osaka, Japan.

¶ To whom correspondence and reprint requests should be addressed.

Schuell, Keene, NH, U.S.A.); rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA, U.S.A.). Purified bovine low- M_r kininogen was a gift from Dr. S. Iwanaga and Dr. H. Kato of the Protein Research Institute, University of Osaka, Osaka, Japan. STZ was a gift from the Upjohn Co. (Kalamazoo, MI, U.S.A.).

Kallikrein assays

Kallikrein-like Tos-Arg-OMe activity was determined as described previously (Margolius *et al.*, 1974). Samples were incubated with Tos-Arg-O[3 H]Me for 30 min at room temperature, and released [3 H]methanol was measured. One esterase unit is defined as the amount of enzyme that hydrolyses 1 μ mol of Tos-Arg-OMe/min at pH 8.0 and 30 °C. Kinin-generating activity (kininogenase activity) was measured by the method of Shimamoto *et al.*, (1979a). Purified bovine low- M_r kininogen (12.5 μ g/tube) was used as a substrate, and generated kinins were measured by a kinin radioimmunoassay (Shimamoto *et al.*, 1978). Kallikrein content in individual skeletal muscles was measured by radioimmunoassay (Shimamoto *et al.*, 1979b), modified from the previous procedure by using a second-antiserum (rabbit anti-sheep globulin) precipitation method.

Preparation of muscle extracts

Male Sprague-Dawley rats (300–400 g) were anaesthetized with diethyl ether and perfused through the left heart with 200–250 ml of 0.9% NaCl. Skeletal muscles were dissected from forelimbs, hindlimbs and paravertebral regions. All muscle tissue was carefully cleaned of fat and connective tissue, and then ground in a food processor. The ground tissue was then homogenized in 0.01 M-phosphate buffer, containing 0.1 M-NaCl (pH 7.0), using a Brinkman Polytron tissue homogenizer (full speed; 30 s). The homogenate was centrifuged at 1000 g for 15 min at 4 °C and the supernatant was retained. Sodium deoxycholate was added at a final concentration of 0.5% to the supernatant, which was then incubated at room temperature for 30 min. This incubation mixture was centrifuged at 23000 g for 20 min at 4 °C in a Beckman JA-20 rotor. The resultant supernatant was stored at –20 °C and used for subsequent purification procedures. The protein concentration of this and all subsequent fractions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Purification of skeletal-muscle kallikrein

Pooled muscle extract was thawed and then centrifuged at 5000 g for 30 min at 4 °C to remove any precipitate that had formed. The supernatant was retained and $(\text{NH}_4)_2\text{SO}_4$ was added to 30% saturation. After centrifugation at 5000 g for 30 min, the resultant supernatant was retained, and to this $(\text{NH}_4)_2\text{SO}_4$ was added at 75% saturation. The pellet obtained after centrifugation at 5000 g for 30 min was dissolved in buffer (0.2 M-NaCl/0.01 M- NaH_2PO_4 , pH 7.0) and dialysed twice against the same buffer for 16 h at 4 °C. Samples were applied to a monoclonal-antibody affinity column (1.5 cm $\times$ 15 cm) equilibrated with buffer (0.2 M-NaCl/0.01 M- NaH_2PO_4 , pH 6.0). The affinity column was prepared by using antibodies from a clonal hybridoma designated 'V $_4$ G $_6$ ', as described previously by

Chao *et al.* (1984). Adsorbed protein was eluted with 0.1 M-acetic acid, pH 3.5 (40 ml/h flow rate). Tos-Arg-OMe activity was determined in eluted fractions. Fractions with peak activity were pooled for enzymic and immunological characterization.

Western-blot analysis

Western-blot analysis of the purified muscle kallikrein, along with purified RUK, was carried out as previously described in detail (Woodley *et al.*, 1985). Briefly, electrophoresis was performed in a linear 7.5–17.5%-polyacrylamide gradient gel containing 0.1% SDS. The proteins were then electrophoretically transferred to nitrocellulose paper (15 V for 18 h at 4 °C). The unstained blots were soaked for 1 h at 40 °C in a buffer containing 5% bovine serum albumin, 0.15 M-NaCl, and 0.01 M-Tris/HCl, pH 7.4, to saturate unoccupied protein-binding sites. Monoclonal antibody (V $_4$ D $_{11}$) was affinity-purified by kallikrein-agarose chromatography (Chao *et al.*, 1983) and iodinated as described previously (Shimamoto *et al.*, 1980). Purified muscle and urinary kallikrein were revealed by autoradiography after direct binding to ^{125}I -labelled monoclonal antibody (Woodley *et al.*, 1985).

Muscle kallikrein levels in normal and diabetic rats

Male Sprague-Dawley rats (200–280 g) were studied. Diabetes was induced in 19 rats by intravenous injection of STZ (60 mg/kg body wt.), which was diluted just before injection in 0.1 M-sodium citrate buffer, pH 4.5. Control rats (20 in all) were not injected. After 14 days, diethyl ether-anaesthetized animals were perfused through the left heart with 200–250 ml of 0.9% NaCl. Individual hindlimb muscles were immediately dissected and processed. Three muscles with different muscle-fibre composition and metabolic properties were chosen. These were soleus, EDL and gastrocnemius. According to the classification of Ariano *et al.* (1973), soleus is a red slow-contracting muscle composed of nearly 85% slow oxidative fibres, with a high content of mitochondrial oxidative-enzyme activity. EDL is a white fast-contracting muscle containing approx. 60% fast oxidative glycolytic fibres and with an intermediate content of oxidative enzyme activity. The gastrocnemius is a complex muscle, containing 60% fast glycolytic fibres with relatively low oxidative enzyme activity.

Muscles were cleaned of fat and connective tissue and chopped finely with scissors. A 1 g sample of gastrocnemius muscle from each rat was used for kallikrein determination. In the case of the soleus or EDL, pairs of muscles from two to three rats were combined to yield 650–800 mg of tissue. Chopped muscles were homogenized, as described above, in 2–3 ml of Dulbecco's phosphate-buffered saline (0.1 M, pH 7.0, Ca^{2+} - and Mg^{2+} -free). Sodium deoxycholate was added to the homogenate (0.5%, w/v), which was then incubated at room temperature for 30 min. The homogenate was centrifuged at 23000 g for 20 min at 4 °C (Beckman JA-20 rotor) and the supernatant, containing solubilized protein, was retained. The pellet was homogenized again in 2–3 ml of buffer, treated with sodium deoxycholate, and centrifuged as during the first homogenization. Supernatants from both extractions were combined and desalted by centrifugal filtration through Sephadex G-25 (Margolius *et al.*, 1974). The filtrate was then dialysed against water for 20 h at 4 °C, a membrane with an M_r

Fig. 1. Immunoreactivity of muscle and urinary kallikrein

The displacement curves of ^{125}I -kallikrein binding to polyclonal anti-kallikrein antiserum in the presence of purified rat skeletal-muscle kallikrein ($\blacktriangle$) or urinary kallikrein ($\circ$) are plotted in log-logit transformation. B/B_0 is the ratio of antibody-bound radioactivity of labelled kallikrein in the presence (B) and absence (B_0) of various amounts of unlabelled muscle or urinary kallikrein.

cut-off of 6000–8000 (Spectrapor membrane tubing; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) being used. Samples were then freeze-dried and reconstituted with 0.1 M-phosphate buffer containing 0.1 M-NaCl, pH 7.0. Kallikrein was measured by radioimmunoassay as described above. Total protein in each sample was measured by the method of Lowry *et al.* (1951).

The levels of muscle kallikrein are expressed as pg/mg of protein. Data are expressed as means $\pm$ S.E.M. The levels of kallikrein in muscle, serum glucose levels, and body and muscle weights of diabetic and control rats were compared by using the two-sided *t* test for unpaired data.

RESULTS

Characterization of muscle kallikrein

Fractions eluted from the monoclonal-antibody affinity column and which showed highest Tos-Arg-OMease activity were pooled. Appropriate dilutions of this enzyme provided a range of concentrations comparable with concentrations of RUK standards used in the radioimmunoassay (Fig. 1). The displacement of ^{125}I -RUK from polyclonal kallikrein antiserum by serial dilutions of RUK or rat muscle enzyme always showed parallel curves (log-logit transformation of data). Therefore, with an antiserum raised to RUK, the muscle and urinary enzymes showed immunological identity. The purified rat muscle enzyme had a Tos-Arg-OMease activity of 291 esterase units/mg of enzyme, as calculated from the enzyme quantity in the direct radioimmunoassay. This enzyme also released kinin from purified bovine low- M_r kininogen at a rate of 4.9 mg of kinin generated/min per mg of kallikrein.

The pH profiles of Tos-Arg-OMease activity of

Fig. 2. Effect of pH on Tos-Arg-OMease activity of rat muscle and urinary kallikrein

Portions (20 μl) of muscle ($\blacktriangle$) or urinary ($\circ$) kallikrein (0.01 esterase unit/ml) in water was added to 30 μl of 0.2 M-Tris/HCl buffer (up to pH 9.0) or 0.2 M-glycine/NaOH buffer (beyond pH 9.0) and left for 10 min. Tos-Arg-O[^3H]Me (3.0×10^4 c.p.m., 10 μl) was added and incubated for 30 min at room temperature. [^3H]Methanol released was measured and enzyme activity was expressed as a percentage of that at pH 9.0.

skeletal-muscle kallikrein and RUK are shown in Fig. 2. Both enzymes showed optimum activity at pH 9.0, with their profiles being virtually superimposed. The effects of trypsin inhibitors on muscle kallikrein and RUK esterase activities are shown in Fig. 3. Aprotinin completely inhibited the esterase activity of both kallikreins at inhibitor concentrations from 17 to 170 kallikrein-inhibitory units/ml. Enzyme activities of both were reduced by high concentrations of SBTI (0.17–1.7 mg/ml), but LBTI affected neither muscle nor urinary kallikrein activity at these concentrations.

Western-blot analysis of skeletal-muscle kallikrein was performed to demonstrate further specific recognition by tissue-kallikrein antibody. After SDS/polyacrylamide-gel electrophoresis and transfer to nitrocellulose paper, blots of muscle and RUK were incubated with ^{125}I -labelled anti-kallikrein monoclonal antibody. Muscle and urinary kallikrein were then revealed by autoradiography (Fig. 4). By this technique, both skeletal-muscle kallikrein and purified RUK produced single bands. The M_r of muscle kallikrein (~ 38000) is slightly greater than that of purified RUK.

Skeletal-muscle-kallikrein levels in normal and diabetic rats

Kallikrein levels were measured in individual hindlimb muscles that have different muscle-fibre profiles (see the Materials and methods section). To determine if the level of skeletal-muscle kallikrein is insulin-regulated, we studied control and diabetic rats. Body weights, serum glucose levels and muscle weights in control and diabetic rats are shown in Table 1. At 14 days after STZ injection, the mean body weight of diabetic rats was significantly lower ($P < 0.01$), and the mean serum glucose level was markedly elevated ($P < 0.001$), compared with normal controls. Muscle weights were also decreased in diabetic

Fig. 3. Effects of trypsin inhibitors on rat muscle kallikrein and RUK activity

Portions (20 μ l) of the enzyme in water (0.01 esterase unit/ml) and 10 μ l of the inhibitor in water (final concn. as indicated) were incubated at 37 °C for 30 min. Then 20 μ l of 0.2 M-Tris/HCl buffer, pH 8.0, was added, mixed, and left for 10 min. Tos-Arg-O[³H]Me (3.0 $\times$ 10⁴ c.p.m., 10 μ l) was added and incubated for 30 min at room temperature. [³H]Methanol release was measured and the enzyme esterase activity was expressed as a percentage of the activity in the absence of inhibitor. Closed symbols represent rat muscle kallikrein and open symbols represent RUK activities. Circles designate activities in the presence of aprotinin (units/ml); triangles, SBTI (mg/ml); squares, LBTI (mg/ml).

rats. The reduction in muscle weight was proportional to the reduction in body weight induced by diabetes.

Among the three types of hindlimb muscles, soleus showed the highest kallikrein level (258 $\pm$ 30 pg/mg of protein). The level in gastrocnemius muscle was lowest (56 $\pm$ 3 pg/mg of protein), being only 20–25% of the soleus concentration. The EDL kallikrein level was intermediate (142 $\pm$ 14 pg/ml of protein). However, no differences were found in any muscle-kallikrein levels between control and diabetic rats.

Fig. 4. Western-blot analysis of RUK and muscle kallikrein

Electrophoresis was performed in a linear 7.5–15% gradient gel containing 0.1% SDS, and proteins were electrophoretically transferred to nitrocellulose. The blots were incubated with ¹²⁵I-labelled monoclonal anti-kallikrein antibody from clone V₄D₁₁. The antibody-bound protein bands were revealed by autoradiography. Purified rat urinary kallikrein is in lane 1, and purified skeletal muscle kallikrein in lane 2. *M_r* used were phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), SBTI (20100) and α -lactalbumin (14400).

DISCUSSION

We have found that rat skeletal muscle contains a tissue kallikrein that is closely similar to urinary kallikrein with respect to *M_r*, recognition by polyclonal and monoclonal kallikrein antibodies, pH optimum and susceptibility to inhibitors. The isolated active enzyme displays both Tos-Arg-OMease and kininogenase activities.

Previous biochemical and histochemical studies have found esterase activity in skeletal muscle or muscle fractions. Goldspink *et al.* (1971) isolated an alkaline proteinase from horse myoglobin. This proteinase had optimal Tos-Arg-OMease activity at pH 8.0–8.5. Both

Table 1. Body weights, serum glucose levels and muscle weights in normal and diabetic rats

Values are means $\pm$ S.E.M.

	Body wt. (g)		Serum glucose (mg/dl)	Muscle wt.		
	Day 1	Day 14		Soleus (mg)	EDL (mg)	Gastrocnemius (g)
Control (n = 20)	244 $\pm$ 6	349 $\pm$ 7	175 $\pm$ 3	315 $\pm$ 17	372 $\pm$ 16	4.71 $\pm$ 0.14
Diabetic (n = 19)	245 $\pm$ 6	299 $\pm$ 6*	527 $\pm$ 16*	258 $\pm$ 12†	282 $\pm$ 11*	3.51 $\pm$ 0.10*

* *P* < 0.001; † *P* < 0.01 compared with control.

Table 2. Muscle kallikrein levels in control and diabetic rats

Values are means $\pm$ S.E.M.; the numbers of observations are given in parentheses.

	Kallikrein (pg/mg of protein)		
	Soleus	EDL	Gastrocnemius
Control	258 $\pm$ 30*† (9)	142 $\pm$ 14† (10)	56 $\pm$ 3 (20)
Diabetic	260 $\pm$ 28*† (7)	144 $\pm$ 13† (6)	69 $\pm$ 7 (19)

* $P < 0.01$ compared with EDL.

† $P < 0.001$ compared with gastrocnemius.

alkaline and neutral esterases have been demonstrated histochemically within muscle fibres of the rat (Holt & Withers, 1957; Romanul, 1964). We do not know the relation between these esterases and muscle kallikrein. However, some interesting comparisons exist between enzymic activities described in various skeletal muscles and the kallikrein levels we have found. Both Goldspink *et al.* (1970) and Pluskal & Pennington (1973) found a higher activity of alkaline proteinase in soleus than in EDL in the rat. Using histochemistry, Romanul (1964) also found the highest esterase activity in soleus, and much less in gastrocnemius. In our study, tissue kallikrein was highest in soleus muscle, intermediate in EDL and lowest in gastrocnemius.

Romanul (1964) histochemically profiled the activities of esterase, mitochondrial oxidative enzymes, phosphorylase and β -hydroxybutyrate dehydrogenase in several muscles. He concluded that esterase activity varied directly with the muscle's capacity for oxidative metabolism of glucose and fatty acids, and inversely with the capacity to utilize stored glycogen. The kallikrein levels we found in the soleus, EDL and gastrocnemius suggest the possibility of a similar relation between muscle kallikrein and muscle metabolism. Since muscles with greater oxidative metabolic activity have a richer vascular supply (Romanul, 1965), and since tissue kallikrein is thought to participate in regulating local blood flow in other organs (Miller & Margolius, 1986), it is possible that kallikrein in skeletal muscle might function to regulate delivery of oxygen and substrates for energy production. Such a function could explain the observation that a tissue kallikrein inhibitor can reduce the uptake of glucose by exercising human forearm (Dietze *et al.*, 1978). A recent study, using the isolated perfused rat heart, demonstrated that kinins influence cardiac muscle metabolism by enhancing oxygen delivery to the contracting muscle (Rosen *et al.*, 1983).

The diabetic state did not produce any change in muscle kallikrein levels in the present study. This is in contrast with observations, by us and others, in other kallikrein-containing tissues. Reductions in renal kallikrein levels and excretion, as well as kallikrein levels of salivary gland and pancreas, have been observed in the rat after 14–28 days of STZ-diabetes (Jaffa *et al.*, 1984; Mayfield *et al.*, 1985; Sakamoto *et al.*, 1985). Together these data support the contention that kallikreins in different tissues may be under different hormonal and physiological regulation (Chao & Margolius, 1983). The

present finding, that the insulin-deficient state did not reduce muscle kallikrein levels, also supports our findings in a recent study of glucose metabolism in isolated skeletal muscle. In the latter study no evidence could be obtained for a role of kallikrein as a direct mediator of insulin action (Shimojo *et al.*, 1984).

At this time the significance and function of skeletal-muscle kallikrein is unknown. It is also unknown if any other components of the kallikrein–kinin system exist in skeletal muscle. Studies can now explore these questions. Changes in the metabolic, enzymic and physiological characteristics of individual muscles are known to follow such events as exercise training or denervation (Edgerton *et al.*, 1969; Burant *et al.*, 1984; Richter *et al.*, 1984). Measurement of muscle kallikrein activity under such conditions, and in relation to disease states, might provide some insight into the functions of tissue kallikrein in skeletal muscle.

We gratefully acknowledge the technical assistance of Ms. Teresa Pickens and the secretarial assistance of Ms. Shirley Tant. We also thank Dr. Donald Miller for his critical review of the manuscript. This work was supported by National Institutes of Health Grants AM-00892, HL-29397 and HL-33552. N.S. was supported by a Clinical Research Fellowship from the College of Graduate Studies, Medical University of South Carolina. R.K.M. was the recipient of a Special Emphasis Research Career Award from NIADDK and NHLBI during these studies.

REFERENCES

- Ariano, M. A., Armstrong, R. B. & Edgerton, V. R. (1973) *J. Histochem. Cytochem.* **21**, 51–55
- Berger, E. A. & Shooter, E. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3647–3651
- Burant, C. F., Lemmon, S. K., Treutelaar, M. K. & Buse, M. G. (1984) *Am. J. Physiol.* **247**, E657–E666
- Cardin, A. D., Witt, K. R., Chao, J., Margolius, H. S., Donaldson, V. H. & Jackson, R. L. (1984) *J. Biol. Chem.* **259**, 8522–8528
- Chao, J. & Margolius, H. S. (1983) *Endocrinology (Baltimore)* **113**, 2221–2225
- Chao, J., Woodley, C. M., Chao, L. & Margolius, H. S. (1983) *J. Biol. Chem.* **258**, 15173–15178
- Chao, J., Chao, L. & Margolius, H. S. (1984) *Biochim. Biophys. Acta* **801**, 244–249
- Chao, J., Chao, L., Swain, C., Tsai, J. & Margolius, H. S. (1987) *Endocrinology (Baltimore)* **120**, 475–482
- Currie, M. G., Geller, D. M., Chao, J., Margolius, H. S. & Needleman, P. (1984) *Biochem. Biophys. Res. Commun.* **120**, 461–466
- Cuthbert, A. W. & Margolius, H. S. (1982) *Br. J. Pharmacol.* **75**, 587–598
- Dietze, G., Wicklmayr, M., Bottger, I. & Mayer, L. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1209–1215
- Dietze, G., Wicklmayr, M., Bottger, I., Schifmann, R., Geiger, R., Fritz, H. & Mehnert, H. (1980) *Agents Actions* **10**, 335–338
- Edgerton, V. R., Gerchman, L. & Carrow, R. (1969) *Exp. Neurol.* **24**, 110–123
- Goldspink, D. F., Harris, J. B., Park, D. C. & Pennington, R. J. (1970) *Enzymol. Biol. Clin.* **11**, 481–490
- Goldspink, D. F., Holmes, D. & Pennington, R. J. (1971) *Biochem. J.* **125**, 865–868
- Holt, S. J. & Withers, R. F. J. (1957) *Proc. R. Soc. London Ser. B* **148**, 520–532

- Jaffa, A. A., Rashid, Z., Pratt, J., Ashford, A. & Bailey, G. S. (1984) *Biochem. Med.* **31**, 42–46
- Jarett, L., Kiechle, F. L. & Parker, J. C. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2736–2741
- Johnson, A. R. (1979) in *Bradykinin, Kallidin and Kallikrein*; (Handb. Exp. Pharmacol. **25** Suppl.) (Erdos, E. G., ed.), pp. 357–399, Springer-Verlag, Berlin, Heidelberg and New York
- Larner, J., Cheng, K., Schwartz, C., Kikuchi, K., Tamura, S., Creacy, S., Dubler, R., Galasko, G., Pullin, C. & Katz, M. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2724–2729
- Leikauf, G. D., Ueki, I. F., Nadel, J. A. & Widdicombe, J. H. (1985) *Am. J. Physiol.* **248**, F48–F55
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Margolius, H. S., Horwitz, D., Geller, R. G., Alexander, Jr., R. W., Gill, J. R., Pisano, J. J. & Keiser, H. R. (1974) *Circ. Res.* **35**, 812–819
- Mayfield, R. K., Margolius, H. S., Bailey, G. S., Miller, D. H., Sens, D. A., Squires, J. & Namm, D. H. (1985) *Diabetes* **34**, 22–28
- Miller, D. H. & Margolius, H. S. (1986) in *Endocrinology* (DeGroot, L. J., ed.), Grune and Stratton, New York, San Francisco and London, in the press
- Nolly, H., Scicli, A. G., Scicli, G. & Carretero, O. A. (1985) *Circ. Res.* **56**, 816–821
- ole-Moi Yoi, O., Seldin, D. C., Spragg, J., Pinkus, G. S. & Austen, K. F. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3612–3616
- Pluskal, M. G. & Pennington, R. J. (1973) *IRCS Libr. Compend.* **1**, 10–30
- Powers, C. A. & Nasjletti, A. (1983) *Endocrinology* (Baltimore) **112**, 1194–1200
- Richter, E. A., Garetto, L. P., Goodman, M. N. & Ruderman, N. B. (1984) *Am. J. Physiol.* **246**, E476–E482
- Romanul, F. C. A. (1964) *Arch. Neurol.* **11**, 355–368
- Romanul, F. C. A. (1965) *Arch. Neurol.* **12**, 497–509
- Rosen, P., Eckel, J. & Reinauer, H. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 1431–1438
- Sakamoto, W., Yoshikawa, K. & Nishikaze, O. (1985) *J. Clin. Chem. Clin. Biochem.* **23**, 521–523
- Seals, J. R. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 6529–6531
- Sealy, J. E., Atlas, S. A., Laragh, J. H., Oza, N. B. & Ryan, J. W. (1978) *Nature (London)* **275**, 144–145
- Shimamoto, K., Ando, T., Nakao, T., Tanaka, S., Sakuma, M. & Miyahara, M. (1978) *J. Lab. Clin. Med.* **91**, 721–728
- Shimamoto, K., Tanaka, S., Nakao, T., Ando, T., Nakahashi, Y., Sakuma, M. & Miyahara, M. (1979a) *Jpn. Circ. J.* **43**, 147–152
- Shimamoto, K., Margolius, H. S., Chao, J. & Crosswell, A. R. (1979b) *J. Lab. Clin. Med.* **94**, 172–179
- Shimamoto, K., Chao, J. & Margolius, H. S. (1980) *J. Clin. Endocrinol. Metab.* **51**, 840–848
- Shimojo, N., Pickens, T. G., Margolius, H. S. & Mayfield, R. K. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 735
- Swift, G. H., Dagorn, J.-C., Ashley, P. L., Cummings, S. W. & MacDonald, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7263–7267
- Woodley, C. M., Chao, J., Margolius, H. S. & Chao, L. (1985) *Biochem. J.* **231**, 721–728

Received 10 September 1986/30 December 1986; accepted 16 January 1987