Biochemical Pharmacology, Vol. 27, pp. 1967–1971

0006-2952/78/0801-1967$02.00/0

ALTERED FUNCTION OF ADENYLATE CYCLASE IN THE MYOCARDIUM OF THE SPONTANEOUSLY HYPERTENSIVE RAT*

RAMESH C. BHALLA and TOM ASHLEY
Department of Anatomy, and Cardiovascular Center, University of Iowa, Iowa City, IA 52242, U.S.A.

(*Received* 20 *October* 1977; *accepted* 12 *January* 1978)

Abstract—The effects of Mg^{2+}, Mn^{2+}, Ca^{2+}, guanylylimidodiphosphate [Gpp(NH)p] and isoproterenol on adenylate cyclase activity in myocardial membrane preparations from spontaneously hypertensive rats (SHR) and Kyoto Wistar normotensive rats (WKY) were compared. The isoproterenol-stimulated adenylate cyclase activity of myocardial membranes from WKY rats responded at a lower threshold (10 vs 200 nM) and with a lower EC 50 (300 nM vs 1 μM) and a higher maximal velocity. When the isoproterenol effect was studied in the presence of 100 nM Gpp(NH)p, the threshold for isoproterenol was similar in SHR and WKY. However, the activity at each dose was significantly lower ($P < 0.05$) in SHR. In the presence of Gpp(NH)p alone, no differences were observed between SHR and WKY. Increasing Mg^{2+} and Mn^{2+} concentrations stimulated adenylate cyclase activity but no differences were observed between SHR and WKY for Mn^{2+} stimulation. However, the SHR myocardial activity was significantly reduced at Mg^{2+} concentrations ranging from 6 to 25 mM. The effect of varying the Mg^{2+} concentration was further tested in the prsence of 1 μM isoproterenol. The adenylate cyclase activity of myocardia of SHR was significantly reduced at Mg^{2+} concentrations between 4 and 24 mM. These o. ervations suggest that sensitivity of the adenylate cyclase to isoproterenol has been decreased in SHR.

Catecholamines have pronounced effects on cardiac contractile force and rate. Some of these effects appear to be mediated by stimulation of the membrane-bound enzyme adenylate cyclase. In the hypertensive state, sensitivity of the myocardial receptors as evidenced by the stimulation of adenylate cyclase has important clinical and pharmacological implications.

The reduced levels of cyclic AMP in the cardiovascular tissues have previously been related to the pathogenesis of hypertension[1, 2]. Analysis of synthetic and degradative mechanisms should provide additional insight into the regulation of cyclic AMP levels. However, the reports on adenylate cyclase and phosphodiesterase show no convincing trend. Adenylate cyclase activity is reported to be increased [3], unchanged [4] or decreased [5] in cardiovascular tissues of spontaneously hypertensive rats (SHR).

We recognized that a more detailed study of the effects of divalent cations, guanylylimidodiphosphate [Gpp(NH)p] and catecholamines on adenylate cyclase activity in the myocardia of SHR and Kyoto Wistar normotensive rats (WKY) is necessary before discrepancies in the literature concerning the activity of this enzyme in the hypertensive state can be evaluated. This paper deals with the effects of Mg^{2+}, Mn^{2+}, Ca^{2+}, Gpp(NH)p and isoproterenol on adenylate cyclase activity in myocardia from SHR and WKY. Our study shows marked differences between SHR and WKY in the adenylate cyclase activity of the myocardia to the β-adrenergic stimulation in the presence and absence of Gpp(NH)p.

*This investigation was supported in part by NIH Grants HL 19027 and HL 14388, and a grant-in-aid from the American Heart Association with funds contributed in part by the Iowa Heart Association.

MATERIALS AND METHODS

Materials. Adenosine 5′-triphosphate (ATP: sodium salt from equine muscle), adenosine 3′,5′-monophosphate, theophylline, Dowex 50W-X8 (200–400 mesh, 8% cross linked, H^+ form) and guanylylimidodiphosphate [Gpp(NH)p] were obtained from Sigma Chemical Co. St. Louis, MO, 2-phosphoenol pyruvate (PEP, trisodium salt) and pyruvate kinase (from rabbit muscle) were obtained from CalBiochem, LaJolla, CA. [^{32}P]ATP (TEA salt, 10–30 Ci/m-mole), [^{3}H]-cyclic AMP (NH_4 salt, 4.0 Ci/m-mole) and Aquasol were purchased from New England Nuclear, Boston, MA.

Adult Kyoto Wistar spontaneously hypertensive rats (SHR) and Kyoto Wistar normotensive rats (WKY), 12 to 16-weeks-old, were used. The SHR maintained at the University of Iowa are inbred descendents of the hypertensive Wistar strain developed by Okamoto and Aoki[6]. The control rats were raised under conditions identical to those used for SHR. Preoperative systolic blood pressures were determined in the unanesthetized state by the tail plethysmographic method using an automated cuff inflator-pulse reading system manufactured by Technilab Instruments. The blood pressure for WKY and SHR was 131 ± 0.2 mm Hg (n = 53) and 186 ± 0.2 mm Hg (n = 53) respectively.

Preparation of myocardial membranes. Rats were sacrificed by decapitation. The ventricles were dissected free of atria and washed with ice-cold homogenizing buffer (0.25 M sucrose, 0.05 M Tris–HCl, pH 7.4) to get rid of blood. The myocardial ventricles were cut into small pieces by scissors and homogenized in 5 vol. of cold homogenizng buffer with an all glass Dounce homogenizer. The homogenate was filtered through two

layers of cheese cloth and centrifuged at 3000 *g* for 15 min to yield pelleted crude plasma membranes. Pelleted membrane fractions were washed once with 0.05 M Tris–Cl, pH 7.4, and the pellet was suspended in homogenizing buffer before being assayed immediately for adenylate cyclase activity.

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Krishna *et al.*[7], with the use of a PEP-pyruvate kinase ATP-regenerating system. The incubation volume of 200 μl contained 200–400 μg protein, 10 mM theophylline, 10 mM PEP, 12.5 I.U./ml of pyruvate kinase, 1 mM cyclic AMP, 2.0 mM ATP-[^{32}P] (5–10 cpm/pmole), 1–24 mM Mg^{2+} or Mn^{2+}, as required by assay conditions, 0.05 M Tris–HCl, pH 7.4, and 20 μl of the drug or reagent tested. The reaction was initiated by the addition of membranes. The incubation time was 5 min, after which the reaction was stopped by the addition of 100 μl of a diluting solution which contained 40 mM ATP and 0.05 M Tris–HCl buffer, pH 7.4, plus 12 mM ^{3}H-cyclic AMP (approximately 17 cpm/nmole) to monitor recovery. The tubes were then immediately placed in a 100° dry bath for 3 min. The cyclic AMP formed was separated by sequential chromatography on columns of Dowex cation exchange resin and aluminum oxide[8]. The activity present in assays conducted without tissue was subtracted from the experimental values. Protein was measured by the method of Lowry *et al.*[9], with bovine serum albumin as the standard. Experiments were repeated at least three times, each experiment being run in triplicate. Statistical analyses were performed by Student's *t*-test for unpaired samples.

RESULTS

Effect of divalent cations. The effects of increasing the Mg^{2+} concentration on adenylate cyclase activity are shown in Fig. 1. It has been a general observation that Mg^{2+}, at concentrations higher than necessary to convert most of the ATP to an ATP–Mg complex, increases basal and hormone-stimulated adenylate cyclase activity. We also observed that the basal and isoproterenol-stimulated adenylate cyclase activity increased with increasing Mg^{2+} concentrations; the activity at 4 mM Mg^{2+} and higher concentrations was significantly greater ($P < 0.05$) than the activity at 2 mM Mg^{2+}, the concentration equimolar with the ATP present in the incubation mixture. The adenylate cyclase activities of WKY and SHR were identical up to 4 mM Mg^{2+}. The adenylate cyclase activity of the myocardia of WKY continued to rise sharply as the Mg^{2+} concentration was elevated above 4 mM, and reached a plateau at 16 mM Mg^{2+}. The adenylate cyclase activity of the myocardia of SHR did not show a steep rise at Mg^{2+} concentrations higher than 4 mM and continued to rise up to 25 mM Mg^{2+}. The adenylate cyclase activity of the myocardia of SHR was significantly reduced ($P < 0.05$) as compared to WKY at 8, 12, 16 and 25 mM Mg^{2+} concentrations.

To test whether the ratio of [ATP]: [Mg^{2+}] or the actual concentrations have an influence on the activity, the experiments were repeated with 1 mM ATP rather than 2 mM ATP and varied concentrations of Mg^{2+}. At equimolar concentrations of ATP and Mg^{2+} no differences were observed between SHR and WKY; however, at higher concentrations the activity of SHR was significantly reduced (data not given). Identical activities were measured for the same ratio of Mg^{2+}: ATP whether a 1 or 2 mM ATP concentration was used, suggesting that the ratio of [ATP]:[Mg^{2+}] may have a greater bearing than the absolute concentrations of ATP and Mg^{2+}.

The effect of varying the Mg^{2+} concentration was further examined in the presence of 1 μM isoproterenol (Fig. 2). Treatment with isoproterenol increased the adenylate cyclase activity which reached a plateau at 12 mM Mg^{2+} both in SHR and WKY. The activity in SHR was

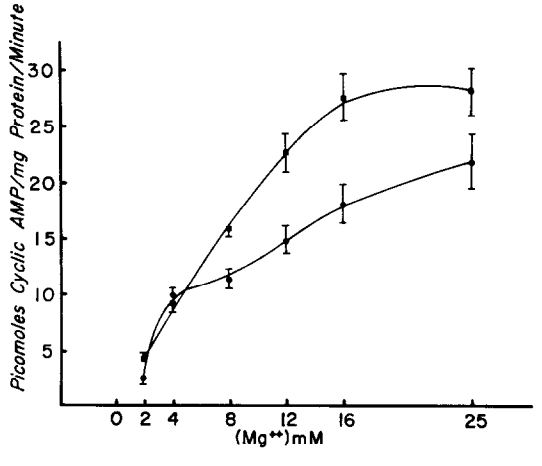

Fig. 1. Adenylate cyclase activity of heart membrane fraction from WKY (■) and SHR (●) in the presence of different concentrations of Mg^{2+}. Each value is a mean ±S.E.M. of four experiments.

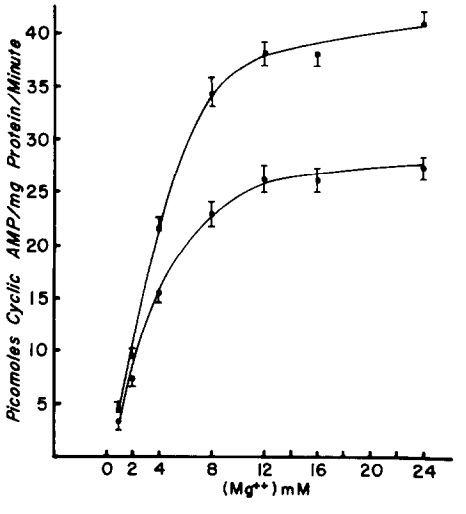

Fig.2. Adenylate cyclase activity of heart membrane fraction from WKY (■) and SHR (●) in the presence of different concentrations of Mg^{2+} and 1 μM isoproterenol. Values are the means ±S.E.M. of three experiments.

significantly decreased ($P < 0.05$) at Mg^{2+} concentrtions between 4 and 24 mM.

Adenylate cyclase activity was also studied at 2 mM ATP with Mn^{2+} substituted for Mg^{2+} at concentrations varying from 1 to 12 mM. Enzyme activity in the WKY appeared slightly greater than in SHR at all concentrations of Mn^{2+} (Fig. 3), but the difference was significant only at the lowest Mn^{2+} concentration (0.25 mM). The adenylate cyclase activity at concentrations up to 4 mM Mn^{2+} was higher than at equimolar Mg^{2+} concentrations in both SHR and WKY. Mn^{2+} in excess of 4 mM was inhibitory.

With 2 mM ATP and 4 mM Mg^{2+} in the assay reaction, Ca^{2+} was added at concentrations from 0.15 to 3.0 mM. Calcium had a biphasic effect on both enzymes (Fig. 4). At low concentrations (0.15 mM), adenylate cyclase activities were stimulated in both SHR and WKY. As the $CaCl_2$ concentration increased up to 1 mM, the enzyme activities were dramatically inhibited with the same apparent K_i, 0.6 mM. Values for SHR were significantly lower than for WKY for most concentrations of Ca^{2+}.

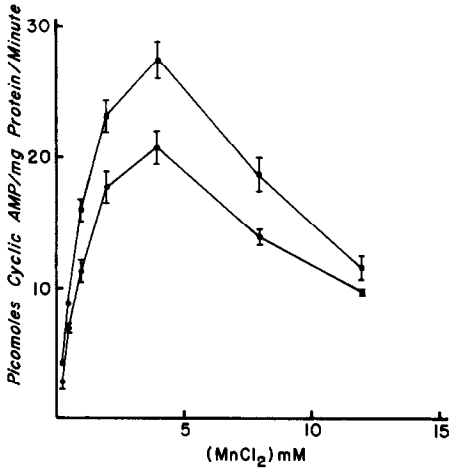

Fig. 3. Myocardial adenylate cyclase activity in the presence of different concentrations of Mn^{2+} from WKY (■) and SHR (●). Each value is a mean ± S.E.M. of three experiments.

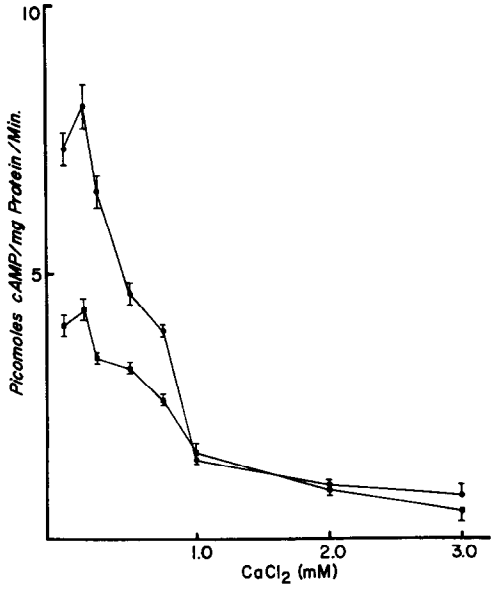

Fig. 4. Adenylate cyclase activity of WKY (●) and SHR (■) heart in the presence of different concentrations of Ca^{2+}. Each value is a mean ± S.E.M. of four experiments.

Effect of isoproterenol and Gpp(NH)p. Marked differences between SHR and WKY were observed in adenylate cyclase stimulation by isoproterenol in the absence and presence of Gpp(NH)p. Treatment of the myocardial membrane preparations with isoproterenol alone revealed that the WKY enzyme responded at a lower threshold (10 vs 500 nM), with a lower EC_{50} (300 nM vs 1 μM), and with a higher maximal velocity than the SHR enzyme (Fig. 5). At every dose of isoproterenol tested, the enzyme activity of SHR was significantly decreased ($P < 0.05$) as compared to that of WKY. When the isoproterenol effect was studied in the presence of 100 nM Gpp(NH)p, the threshold for isoproterenol decreased for SHR and was approximately equal to WKY. However, the activity at each dose of isoproterenol was significantly lower ($P < 0.05$) in SHR (Fig. 6) in the presence of Gpp(NH)p. Gpp(NH)p alone stimulated adenylate cyclase activity. However, no differences were observed between SHR and WKY (Fig. 7). Addition of 100 nM isoproterenol to the various concentrations of Gpp(NH)p potentiated this response (Fig. 8). Furthermore, addition of isoproterenol resulted in significant differences, with decreased activity in SHR ($P < 0.05$) at each dose of Gpp(NH)p. These observations suggest that the sensitivity of adenylate cyclase to isoproterenol has been altered (decreased) in SHR.

DISCUSSION

The importance of cyclic nucleotide aberrations and the role of sensitivity of adenylate cyclase to isoproterenol stimulation in the cardiovascular tissues of spontaneously hypertensive (SHR) and Kyoto Wistar normotensive control rats (WKY) have been studied. β-Adrenergic receptors in my-

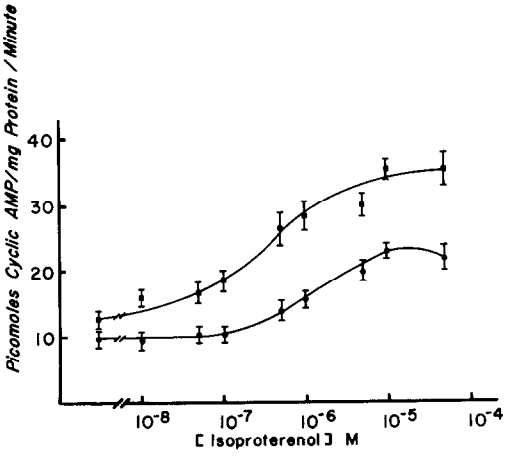

Fig. 5. Myocardial adenylate cyclase activity in the presence of different concentrations of isoproterenol from WKY (■) and SHR (●). Each value is a mean ± S.E.M. of four experiments.

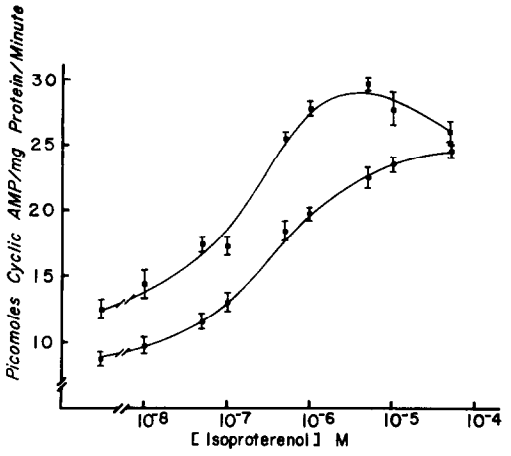

Fig. 6. Adenylate cyclase activity of myocardial membrane from WKY (■) and SHR (●) in the presence of different concentrations of isoproterenol and 100 μM Gpp(NH)p. Each value is a mean ± S.E.M. of three experiments.

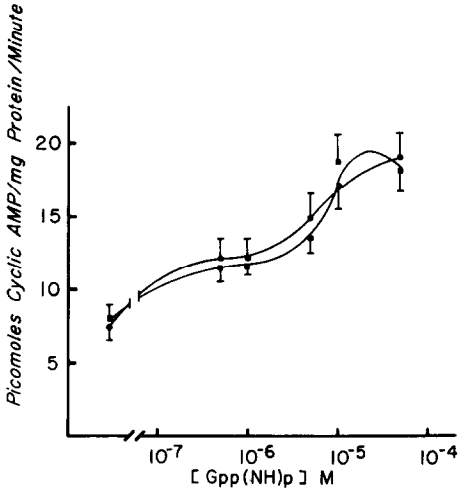

Fig. 7. Adenylate cyclase activity of myocardial membrane from WKY (■) and SHR (●) in the presence of different concentrations of Gpp(NH)p. Each value is a mean ± S.E.M. of four experiments.

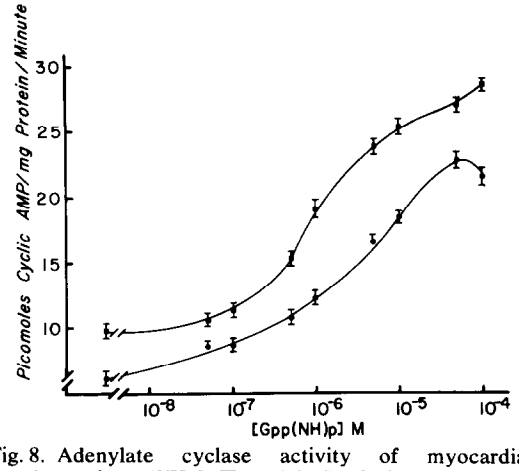

Fig. 8. Adenylate cyclase activity of myocardial membrane from WKY (■) and SHR (●) in the presence of different concentrations of Gpp(NH)p and 100 nM isoproterenol. Each value is a mean ± S.E.M. of four experiments.

ocardial tissue mediate the inotropic response to catecholamines, in part, by the activation of membrane-bound adenylate cyclase. One of the important findings in this study, the decreased sensitivity of the adenylate cyclase to catecholamine, has also been observed by Amer *et al.*[4, 10, 11] and Triner *et al.*[3].

Studies by Rodbell *et al.* [12] on glucagon sensitive adenylate cyclase of rat liver plasma membranes reveal that the enzyme system contains at least three sites through which ligands interact and affect enzyme activity: (1) the hormone receptor site (or discriminator); (2) the nucleotide site (amplifier) which reacts preferentially with GTP or Gpp(NH)p which also appears to react with high concentrations of ATP; and (3) the catalytic site, which reacts with Mg-ATP, the active form of substrate. These observations point out the complexity of the adenylate cyclase system and reveal that hormone sensitivity could be lost by alterations in any one or all of the units of the enzyme. As shown in Fig. 7, Gpp(NH)p had a comparable effect in activating adenylate cyclase from SHR and WKY, suggesting that the nucleotide site may not have been altered in SHR. It is likely that sensitivity of the adenylate cyclase to β-adrenergic stimulation has been reduced in the myocardia of SHR, probably due to either the altered function of β-receptor or its coupling to the adenylate cyclase, or to both. It could also result from the reduced number of total molecules of adenylate cyclase enzyme in SHR. It is postulated that these changes observed in the myocardium may also be occurring in the periphery. However, we were unable to get consistent and reproducible results for isoproterenol stimulation of adenylate cyclase in the vascular smooth muscle of SHR and WKY. Therefore, it was not possible to test this hypothesis.

In general, in disease conditions the most common cyclic nucleotide abnormality observed has been the abnormal sensitivity of the adenylate cyclase to the appropriate stimuli. This is not unexpected because of the fact that this enzyme represents the interface at which the hormones induce biochemical responses in the target cell. Examples of reduced adenylate cyclase sensitivity in disease conditions include loss of sensitivity to catecholamines in asthma [13, 14], muscle dystrophy [15] and psoriasis [16], immunodeficiency [17] and loss of sensitivity to a variety of stimuli in cancer cells [18–22].

It has been demonstrated repeatedly that exposure to catecholamines of cells and tissues possessing beta-receptors results in a diminished cyclic AMP generation in response to subsequent beta-adrenergic challenge [23–30]. Among the various explanations that have been proposed to account for this phenomenon are: (1) prolonged beta-adrenergic stimulation results in elevated intracellular cyclic AMP levels which induce an increase in intracellular phosphodiesterase activity, thereby diminishing the net levels of cyclic AMP [25]; and (2) there is a loss of beta-adrenergic binding sites in sensitized cells [28]. Observations made in this laboratory indicate an increase in high K_m, membrane associated phos-

phodiesterase activity in the vascular and cardiac muscle of SHR as compared to WKY (unpublished observations). The increased phosphodiesterase activity may in part be responsible for the reduced sensitivity of adenylate cyclase in SHR. It has been postulated [31] that, in SHR, excessive hormonal secretion or nervous tone at some early stage of disease development, possibly in an effort to control a deteriorating situation, may result in the desensitization of the adenylate cyclase to further stimulation. In recent years, excellent work carried out by Mukherjee *et al.* [32] has demonstrated a direct approach to the study of the binding of stimuli and receptor by using the specific β-receptor antagonist, (-)-[^{3}H] alprenolol. Experiments to provide a definitive answer as to whether the binding of stimuli to the receptor or the quantity of adenylate cyclase is reduced in SHR are in progress.

Acknowledgement—Mrs. Sue Johnson participated in some of the initial experiments. We wish to thank her for her useful suggestions and expert technical assistance.

REFERENCES

1. M. S. Amer, in *Cyclic Nucleotides in Disease* (Ed. B. Weiss) p. 133. University Park Press, Baltimore (1975).
2. S. Ramanathan, S. Shibata, T. K. Tashahi and R. N. Ichord, *Biochem. Pharmac.* **25**, 233 (1976).
3. L. Triner, Y. Vulliemoz, M. Verosky and W. Manger, *Biochem. Pharmac.* **24**, 743 (1975).
4. M. S. Amer, *Science, N.Y.* **179**, 807 (1973).
5. S. Ramanathan and S. Shibata, *Blood Vessels* **11**, 312 (1974).
6. K. Okamoto and K. Aoki, *Jap. Circul. J.* **27**, 282 (1963).
7. G. Krishna, B. Weiss and B. Bowdie, *J. Pharmac. exp. Ther.* **163**, 379 (1968).
8. Y. Salomon, C. Londos and M. Rodbell, *Analyt. Biochem.* **58**, 541 (1974).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. M. S. Amer, A. W. Gomoll, J. W. Perhach, Jr., H. C. Ferguson and G. R. McKinney, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4930 (1974).
11. M. S. Amer, N. Doba and D. J. Reis, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2135 (1975).
12. M. Rodbell, M. C. Lin, Y. Salomon, C. Londos, J. P. Harwood, B. R. Martin, M. Randell and M. Berman, *Adv. Cyclic Nucleotide Res.* **5**, 3 (1975).
13. P. Fireman, *Int. Arch. Allergy* **45**, 123 (1973).
14. R. A. Berstein, L. Linarelli, M. A. Facktor, G. A. Friday, A. L. Drash and P. Fireman, *J. Lab. clin. Med.* **80**, 772 (1972).
15. S. Mawatari, A. Tagaki and L. P. Rowland, *Archs Neurol.* **30**, Chicago 90 (1974).
16. M. S. Amer and G. R. McKinney, *Life Sci.* **13**, 753 (1973).
17. R. G. Kemp, Y.-C. Huang and R. J. Duquesnoy, *J. Immun.* **111**, 1855 (1973).
18. H. Mukergee and A. Goldfeder, *Fedn Proc.* **33**, 1861 (1974).
19. V. Tomasi, A. Rethy and A. Trevisani, *Life Sci.* **12**, 145 (1973).
20. W. Grimm and F. Marks, *Hoppe-Seyler's Z. physiol. Chem.* **355**, 1198 (1974).
21. H. Boyd and T. J. Martin, *Clin. Sci. molec. Med.* **47**, 20 (1974).
22. A. K. Verma and A. W. Murray, *Cancer Res.* **34**, 3408 (1974).
23. M. H. Makman, *Fedn Proc.* **30**, 458 (1971).
24. T. J. Franklin and S. J. Foster, *Nature New Biol.* **246**, 146 (1973).
25. J. P. Schwartz and J. V. Passoneau, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3844 (1974).
26. E. Remold-O'Donnell, *J. biol. Chem.* **249**, 3615 (1974).
27. J. DeVallis and G. Brooker, *Science, N.Y.* **186**, 1221 (1974).
28. C. Mukherjee, M. G. Caron and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1945 (1975).
29. R. Ho and E. W. Sutherland, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1773 (1975).
30. T. J. Franklin, W. P. Morris and P. A. Twose, *Molec. Pharmac.* **11**, 485 (1975).
31. M. S. Amer, *Life Sci.* **17**, 1021 (1975).
32. C. Mukherjee, M. G. Caron, M. Coverstone and R. J. Lefkowitz, *J. biol. Chem.* **250**, 4869 (1975).