Acknowledgements—We thank Matthew Woodland for his technical assistance, Dr. M. A. Ondetti (Squibb Institute for Medical Research) for the gift of CCK-8, and Dr. P. O'Connell (Upjohn Co.) for the gift of TMB-8.

Department of Physiology
University of Adelaide
Adelaide, South Australia 5000

KARIN A. TENNES*
JENNIFER A. KENNEDY
MICHAEL L. ROBERTS

REFERENCES

1. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
2. S. Cockcroft, J. P. Bennett and D. Gomperts, *Biochem. J.* **200**, 501 (1981).
3. R. L. Bell and P. W. Majerus, *J. biol. Chem.* **255**, 1790 (1980).
4. K. A. Tennes and M. L. Roberts, *Aust. J. exp. Biol. med. Sci.* **59**, 791 (1981).
5. J. A. Williams, *Am. J. Physiol.* **238**, G269 (1980).
6. I. F. Charo, R. D. Feinman and T. C. Detwiler, *Biochem. biophys. Res. Commun.* **72**, 1462 (1976).
7. S. Rittenhouse-Simmons and D. Deykin, *Biochim. biophys. Acta* **543**, 409 (1978).
8. J. O. Shaw, *Prostaglandins* **21**, 571 (1981).
9. R. T. Jensen and J. D. Gardner, *Fedn Proc.* **40**, 2486 (1981).
10. J. A. Williams, P. Cary and B. Moffat, *Am. J. Physiol.* **231**, 1562 (1976).
11. C. Labarca and K. Paigen, *Analyt. Biochem.* **102**, 344 (1980).
12. G. W. Snedecor and W. G. Cochran, *Statistical Methods*, 6th Edn. Iowa State University Press, Ames, IA (1967).
13. A. S. Fairhurst, M. L. Whittaker and F. J. Ehlert, *Biochem. Pharmac.* **29**, 155 (1980).
14. J. S. Aguilar, M. Criado and E. De Robertis, *Eur. J. Pharmac.* **68**, 317 (1980).
15. E. H. Mürer, G. J. Stewart, K. Davenport and E. Siojo, *Biochem. Pharmac.* **30**, 523 (1981).
16. W. J. Taylor, A. Wolf and J. M. Young, *Br. J. Pharmac.* **71**, 327 (1980).

* Author to whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 32, No. 13, pp. 2118–2120, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

Adenylate cyclase activity of circular and longitudinal muscle layers of rat myometrium*

(*Received* 18 *October* 1982; *accepted* 17 *January* 1983)

The uterus is composed of two smooth muscle layers, an outer longitudinal and an inner circular layer which are separated by a well-developed vascular bed [1]. Most physiological studies that have measured effects of catecholamines on uterine contractility used the longitudinal axis, although Nesheim [2] found that the circular and longitudinal layers responded quite differently. He found the circular layer was less sensitive to isoprenaline and, since then, the circular and longitudinal layers have been shown also to differ in their motility patterns [3] and in their sensitivities to indomethacin and prostaglandins [4].

We separated the longitudinal from the circular muscle layer of the rat myometrium using two different methods. Adenylate cyclase activity of the outer longitudinal layer was greater and more responsive to isoproterenol than that of the inner circular layer. Since cAMP mediates the function of the beta-adrenergic receptor in the smooth muscle cell [5], there are important anatomical differences in the distribution of adenylate cyclase in the myometrium.

Materials and methods

The circular and longitudinal layers of the myometrium were separated either by mechanical or enzymatic manipulations.

Mechanical separation. The uteri of eight Sprague–Dawley rats (250 g) were removed and placed in warm (25°) Hanks' solution (HS), without Ca^{2+} or Mg^{2+}, buffered with Na^+, K^+ phosphate (1.5 mM) and PIPES† (5 mM), gassed with carbogen (95% O_2, 5% CO_2), and adjusted to pH 7.5 with NaOH. The uteri were cut open, and the endometrium was scraped off with a microscope slide. The circular (inner) layer was then separated from the longitudinal (outer) layer with the edge of a microscope slide firmly applied on the luminal side and moved from the cervical to the fallopian end of each horn. The different layers were transferred to 5 vol. of cold (4°) adenylate cyclase homogenization buffer (CHB): 0.05 M Na^+-HEPES, 0.001 M EGTA, 10% dimethyl sulfoxide, pH 7.6. The tissue was homogenized at 4° with a polytron PT10 (Brinkmann Instruments, Westbury, NY) for 30 sec at a setting of 4.5. The homogenates were filtered through glass wool and centrifuged at 20,000 *g* for 30 min at 4°. The pellets were washed twice, resuspended in 5 vol. of CHB, and used fresh.

Enzymatic separation. The uteri of twelve Sprague–Dawley rats (250 g) were removed and placed in warm (25°) HS. Six uteri were tied at the cervix and at both fallopian ends with surgical silk while the rest were turned inside out and then tied in an identical manner. Each group of uteri was transferred to Erlenmeyer flasks containing 1 ml of HS with 0.02% EDTA, 0.30 mg/ml trypsin (Type III from bovine pancreas, Sigma Chemical Co., St. Louis, MO), 0.30 mg/ml collagense (Type II, 320 units/mg, Sigma), and 0.10 mg/ml deoxyribonuclease I (bovine pan-

* Supported by research funds from the Veterans Administration. M. F. was supported by Fellowship funds provided by Le Fonds de la Recherche en Sante du Quebec.

† Abbreviations: PIPES, piperazine-*N*,*N*′-bis[2-ethane sulfonic acid] disodium salt; EDTA, [ethylene dinitrilo]-tetraacetic acid, disodium salt; EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

creas, Sigma). The flasks were stoppered, gassed with CO_2:O_2 (5%:95%), and incubated in a shaking bath at 37° for 30 min. The suspension was filtered through a nylon mesh, the filtrate was discarded, and the undigested tissue that was retained by the filter was incubated for 1 hr with fresh enzyme preparation. The suspension was filtered again, and the undigested tissue was incubated for an additional hour with fresh enzyme. The filtrate was diluted to 40 ml with HS at 25° and centrifuged at 500 *g* for 15 min; the pellet was kept on ice. The cells liberated from the last digestion were processed as described above. The two cell pellets were combined and resuspended in 30 ml of CHB at 4°. The enzymatically dispersed cells were broken in a Dounce homogenizer and centrifuged at 4° at 20,000 *g* for 30 min. The pellet was washed twice and then resuspended in CHB at 4° and used fresh.

Determination of adenylate cyclase activity. Adenylate cyclase activity was determined as the enzymatic conversion of [^{32}P]ATP to [^{32}P]cAMP in 5 min according to our previously described method [6]. Protein concentration was determined by the method of Lowry *et al.* [7] using bovine serum albumin as standard.

Results and discussion

Either method separated the myometrium into approximately equal fractions of inner (circular) and outer (longitudinal) layers. Based on total protein content, the distribution (mean ± S.E.M.) in five separate preparations (three mechanical and two enzymatic separations) was: 53.6 ± 13.6% inner layer and 46.4 ± 13.4% outer layer. Despite the equal distribution of protein, the adenylate cyclase activity of the longitudinal muscle layer was greater than that of the circular layer regardless of the method of separation. Consequently, the results obtained using the two methods were combined for subsequent analysis. The enzyme activity of the longitudinal layer showed a large response to stimulation by GTP compared to that of the circular layer (Fig. 1). Stimulation of adenylate cyclase activity of either layer by isoprenaline required the addition of GTP as described previously for the whole myometrium [7]. Besides greater adenylate cyclase activity, the longitudinal layer of the myometrium showed a response to isoprenaline (in the presence of GTP) that was three times greater than that of the circular layer. The adenylate cyclase activity of both layers showed the same sensitivity to stimulation by isoprenaline, and half-maximal activation (EC_{50}) occurred at a concentration of 5×10^{-7} M (Fig. 2). The present results are in perfect agreement with contractile studies in the rabbit [8, 9] and in the rat [10]. In both species, the circular layer of the uterus was less sensitive to beta-adrenergic agonists than the longitudinal layer. Indeed, adenylate cyclase is believed to mediate the relaxation induced by stimulation of the beta-adrenergic receptor [5]. It has also been found that the two layers were different in other respects including the modulation of their alpha- and beta-adrenergic catecholamine receptors by sex steroid hormone [11]. Moreover, the isoprenaline sensitivity of adenylate cyclase in outer muscle layers obtained by trypsin-collagenase treatment demonstrates the resistance of the beta-adrenergic receptor to proteolysis in the intact smooth muscle cell.

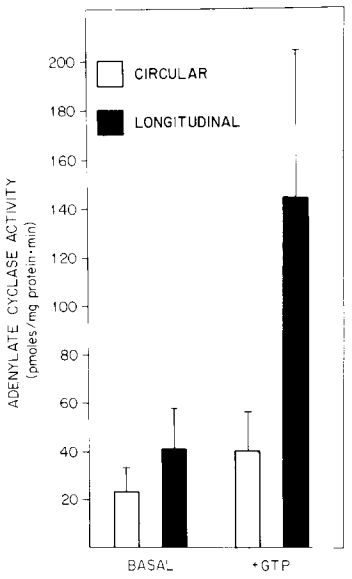

Fig. 1. Stimulation by GTP of adenylate cyclase from uterine circular and longitudinal muscle layers. Adenylate cyclase activity was determined as the conversion of [^{32}P]ATP to [^{32}P]cAMP. Enzyme activity in the absence (basal) or presence of 300 μM GTP was measured using the muscle layers obtained by the two methods described in Materials and Methods. Values are the mean ± S.E.M. of the results obtained with two preparations of each of the two methods of separation (N = 4).

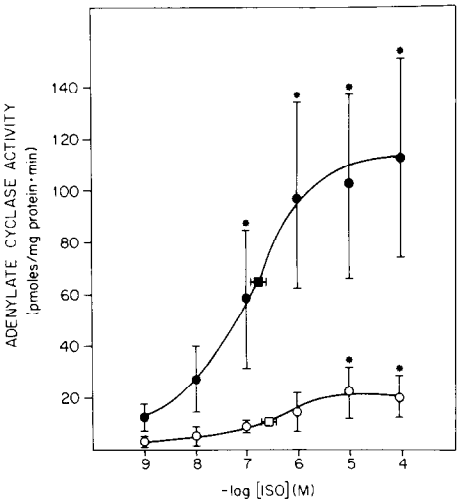

Fig. 2. Beta-adrenergic receptor activation of adenylate cyclase from separated circular (○) and longitudinal (●) layers of the myometrium. Enzyme from the layers was stimulated by increasing the isoprenaline (ISO) concentration from 1 nM to 0.1 mM in the presence of 300 μM GTP. Activity in the presence of GTP alone was subtracted in each case. The results are the mean ± S.E.M. of those obtained using two enzymatically separated and two mechanically separated preparations. Key: (■) and (□) are the mean ± S.E.M. of the EC_{50} for ISO in the four separate preparations; and (*) significantly different ($P < 0.05$) from activity in the presence of GTP alone.

The anatomical complexity of the distribution of beta-adrenergic receptor-dependent adenylate cyclase activity that we have described might explain, in part, discrepancies that have been found between adrenergic receptor concentrations and physiologic responsiveness in the myometrium [12, 13]. The former, determined by specific radioligand binding methods, utilizes subcellular fractions derived from whole muscle strips. Consequently, the methods we have described may prove useful in such studies.

In summary, the longitudinal (outer) and circular (inner) layers of the rat myometrium were separated, and adenylate cyclase properties of each layer were determined. It was found that the longitudinal layer was enriched in adenylate cyclase and was more responsive to GTP and beta-

adrenergic stimulation by isoprenaline. These results are a biochemical explanation for increased beta-adrenergic receptor response of the longitudinal layer found in contractile studies.

Biochemical Pharmacology Laboratory, and the UCLA-SFVP Department of Medicine
Veterans Administration Medical Center
Sepulveda, CA 91343, *U.S.A.*

MICHEL FORTIER
J. FREDERICK KRALL*

REFERENCES

1. T. Kawarabayashi and J. M. Marshall, *Biology Reprod.* **24**, 373 (1981).
2. B-I. Nesheim, *Acta pharmac. tox.* **31**, 296 (1972).
3. T. Kawarabayashi and T. Osa, *Jap. J. Physiol.* **26**, 403 (1976).
4. G. F. Anderson, T. Kawarabayashi and J. M. Marshall, *Biology Reprod.* **24**, 359 (1981).
5. J. F. Krall, M. Fortier and S. G. Korenman, in *Smooth Muscle Cyclic Nucleotide Biochemistry. Biochemistry of Smooth Muscle* (Ed. N. L. Stephens), in press.
6. J. F. Krall, S. C. Leshon, M. Frolich and S. G. Korenman, *J. biol. Chem.* **256**, 5436 (1981).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193** 265 (1951).
8. B-I. Nesheim, *Acta pharmac.* **34**, 295 (1974).
9. B-I. Nesheim, J-B. Osnes and I. Oye, *Br. J. Pharmac.* **53**, 403 (1975).
10. T. Osa and T. Katase, *Jap. J. Physiol.* **25**, 153 (1975).
11. T. Osa and T. Fugino, *Jap. J. Physiol.* **28**, 197 (1978).
12. J. M. Roberts, P. A. Insel, R. D. Goldfin and A. Goldfein, *Nature, Lond.* **270**, 624 (1977).
13. G. Kunos, B. Hoffman, Y. N. Kwok, W. H. Kan and L. Mucci, *Nature, Lond.* **298**, 254 (1979).

* To whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 32, No. 13, pp. 2120–2122, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

Covalent binding of the proximate carcinogen, 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) to rat liver cytosolic protein via 7-HMBA sulphate

(*Received* 6 *October* 1982; *accepted* 10 *February* 1983)

7 - Hydroxymethyl - 12 - methylbenz[*a*]anthracene (7-HMBA), a potent carcinogen [1] and a major metabolite of 7,12-dimethylbenz[*a*]anthracene (DMBA) in rat liver [2, 3], has very recently been demonstrated to be activated by a dialysed hepatic soluble supernatant fraction (S105) in the presence of a PAPS-generating system to a potent mutagen towards *Salmonella typhimurium* TA 98 [4]. The active metabolite, 7-HMBA sulphate, has been isolated as a sodium salt from the incubation mixture and found to show a potent mutagenicity to the bacteria in the absence of S105 and the PAPS-generating system [4]. Moreover, the bacterial mutagenicity of 7-HMBA or DMBA by a rat liver supernatant fraction (S9) fortified with NADPH- and PAPS-generating systems was much higher than that by S9 fortified with the NADPH-generating system alone [4], strongly suggesting that metabolically formed epoxides of 7-HMBA [5] or DMBA [6] are less important than 7-HMBA sulphate with regard to their mutagenicity.

Sulphotransferase-mediated activation of proximate carcinogens was first proposed by Miller and Miller [7] with *N*-OH-FAA and *N*-OH-MAB, a putative oxidative metabolite of MAB. They have demonstrated that *N*-OH aromatic amine derivatives bind to proteins and nucleic acids in the presence of S105 and PAPS, but failed to detect the active sulphates from the biological systems because of their suggested instability.

7-HMBA sulphate was readily inactivated by S105 in the presence of glutathione to form a non-mutagenic conjugate which was identified as *S*-(12-methylbenz[*a*]anthracene-7-methyl)glutathione [8]. The present investigation deals with the covalent binding of 7-HMBA to S105 protein in the presence of a PAPS-generating system and with the isolation and identification of three carcinogen–amino acid adducts from the protein.

7-HMBA (1 μmole in 1 ml dimethylsulphoxide) was incubated at 37° for 20 min with S105 (35 mg protein, equivalent to 500 mg liver) from male Wistar rats (100–120 g) in the presence of ATP, sodium sulphate (50 μmoles each), magnesium chloride (30 μmoles) and EDTA (1 μmole) in a final volume of 10 ml of 0.1 M phosphate buffer, pH 7.4. Protein was precipitated from the mixture by the addition of an equal volume of 10% trichloracetic acid (TCA), collected by centrifugation, washed successively twice with 5% TCA, acetone and ethanol (10 ml each), dried *in vacuo*, dissolved in 2 N NaOH (2.5 ml), washed with ether, bubbled with nitrogen, and diluted with water (to a final volume of 10 ml). The alkaline solution of the isolated protein showed an intense fluorescence emission spectrum with peak maxima at 408 and 426 nm (relative intensity 1.15 : 1) when irradiated by a 361 nm ray for excitation. The spectrum was superimposable on that recorded with 49.5 nmoles 7-HMBA in the same volume of 0.5 N NaOH. The isolated protein, however, showed no appreciable fluorescence spectrum either when ATP or sodium sulphate was omitted from the incubation mixture or when boiled S105 was used. Data strongly suggest that the hepatic protein