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THERMAL ALTERATION IN RECEPTOR ACTIVITY OF THE RAT FUNDAL STRIP[1, 2]

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ABSTRACT

FLEISCH, JEROME H. AND S. EHRENPREIS: Thermal alteration in receptor activity of the rat fundal strip. J. Pharmacol. Exp. Therap. **162**: 21–29, 1968. The objective of this study was to use a physical method in an attempt to characterize the molecular nature of drug receptors *in situ*. The isolated rat stomach fundus strip was heated at various temperatures for various times. Dose-response curves to KCl and a number of receptor agonists were determined before and after heating. The experimental dose-response data were obtained after reequilibrating the tissue at the control temperature (37.5°C). Optimal conditions for achieving differential thermal effects were 47°C for 20 min. KCl-induced contractions were unaffected, as were those to acetylcholine and bradykinin. A marked decrease in slope of the serotonin dose-response curve was produced. Although the maximum contraction height could be attained, this required a 1250-fold higher concentration than the control. Dose-response curves to angiotensin and vasopressin were affected to the greatest degree, showing a marked decrease in slope and a greatly depressed maximum contraction height. The simplest interpretation for the depressed responses is that heat caused an alteration in the structure of the active site on the various receptors. The comparatively mild conditions producing these effects suggest that the heat-labile receptors are composed entirely or in part of protein molecules which undergo irreversible heat denaturation.

In recent years, a number of investigators have attempted to obtain information concerning the molecular nature of pharmacologic receptors. Woolley (1958, 1959) originally suggested that receptors for serotonin and acetylcholine (ACh) are simple lipids which could be extracted from hormone-susceptible cells. Subsequently, Woolley and Gommi (1964, 1966) showed that neuraminidase and ethylenediamine tetraacetic acid could produce a selective abolition of the response to serotonin, leading to the proposal that gangliosides, rather than simple lipids, play an essential role in the action of serotonin. Chagas and co-workers (Chagas, 1959; Hasson and Chagas, 1961) considered that a hyaluronic acid-type molecule isolated from electric organ of electric eel showed ACh receptor activity. Ehrenpreis (1960) reported the isolation of a phospholipoprotein from the same tissue by means of precipitation with *d*-tubocurarine. This protein appeared to possess ACh receptor properties, although subsequent studies (Ehrenpreis, 1962) showed that this component most likely did not function as this receptor. Turpajev *et al.* (1963) also suggested that the cholinergic receptor is a protein, using the frog heart as a source of receptor material. Župančič (1953, 1967), Wurzel (1967), Belleau (1964, 1967) and Ehrenpreis (1967a,b) have considered that the cholinesterase molecule performs a primary role in cholinergic receptor activity. Dikstein and Sulman's (1965) evidence that phospholipids may be extracted from the ACh and adrenergic receptors, as demonstrated by the action of acetone on various tissues, has not been con-

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firmed in this laboratory (Ehrenpreis *et al.*, 1968).

The above-mentioned studies utilized various chemical approaches in attempting to characterize receptor substances. It is conceivable that receptors might be altered *in situ* by physical means, thereby providing information of a different nature concerning their general molecular properties and enabling a decision to be made whether receptors are proteins or some other type of macromolecule. Such information would greatly facilitate future receptor isolation procedures. Temperature is perhaps the most obvious physical means which can be used for this purpose and the objective of this paper is to describe the effect of heating smooth muscle (rat stomach fundus) on its subsequent response to a variety of agonists. Alteration in responsiveness may be considered to reflect the degree of thermal stability of the receptor systems[5] which are involved in the action of these agonists.

In order to be certain that the data from this type of experiment are valid, it must be demonstrated that any observed effect arises specifically from the selective alteration of a particular receptor system. This is a necessary requirement because nonreceptor portions of the active muscle membrane and contractile elements are undoubtedly composed, at least in part, of thermolabile protein material. In order to distinguish between temperature effects upon these extrareceptor elements and on the receptors themselves, we determined the effect of the heating procedure on KCl-induced contractions, since the responses to KCl are not involved in any major way with known receptor mechanisms. Conditions were ultimately found under which effects on receptor agonists could be completely dissociated from those concerned with the contraction of the tissue by KCl.

Serotonin and angiotensin are known to act indirectly on some tissues by releasing ACh *via* stimulation of ganglionic or other neuronal sites (Gaddum and Picarelli, 1957; Khairallah and Page, 1961). It is conceivable that these sites could be altered by heat, and thus it was necessary to ascertain whether ACh release constitutes a major aspect of the activity of these compounds on the rat stomach. It may be noted that Vane (1957) and Offermeier and Ariëns (1966) have already presented considerable evidence that serotonin acts directly on this tissue.

[5] By "receptor systems" we mean not only the specific macromolecules which directly interact with the agonists, but also those components, if any, that intervene in the steps linking the receptor to the contractile elements *via* the conducting membrane.

METHODS AND MATERIALS. The isolated rat stomach fundal strip, a tissue known to have specific receptors for a number of compounds, was used in these studies. Stomach strips from 200-g male Sprague-Dawley rats were set up essentially as described by Vane (1957). The best results were obtained with strips of 3- to 4-mm width, which attained a length of at least 7.5 cm after 1 to 2 hr of equilibration at 37.5°C. Contractions were recorded with a Phipps and Bird linear motion transducer (model ST2) connected to a Sanborn polygraph. The use of a sensitive isotonic recording lever was of paramount importance since, after heating, the strip was in a weakened state. If it was forced to pull against a heavy lever, a complete loss of responsiveness to all agonists invariably resulted. If, however, a frictionless, light-weighted (1.4 g counterweight) system was utilized, repeated contractions could be obtained for at least 4 hr after the treatment. The tissue was bathed in Krebs-bicarbonate buffer (Umbreit, 1957) at a pH of 7.4 and gassed with 95% oxygen–5% carbon dioxide.

In each instance, dose-response curves to KCl and one specific agonist (ACh, serotonin, bradykinin, angiotensin or vasopressin) were obtained at 37.5°C. The temperature was increased by 1-degree intervals and maintained by means of a Haake constant-temperature unit for various times at the given temperature. The temperature of the bathing fluid was monitored by placing a thermometer in close proximity to the stomach strip. In our set-up it took 5 min to raise the bath temperature from 37.5°C to 47°C, the temperature ultimately used in this study. After the given time interval, the system was rapidly cooled to 37.5°C by replacing the high-temperature fluid in the organ bath with buffer at 25°C and simultaneously adding cooled water to the remainder of the circulating system.

As the temperature approached 47°C, the tissue contracted spontaneously. In most instances the tissue relaxed during heating and then went into contracture about 60 min after the end of the heating period. The contracture was generally eliminated either by manually stretching for 45 to 75 sec once every 5 to 10 min or by adding a concentration of KCl which gave about 50% of the control maximum con-

traction height. This was followed by wash and restretching for 60 to 75 sec. When the base line returned to the control level, the second set of dose-response curves was obtained. This usually required approximately 2 to 2½ hr from the time the organ bath was brought back to the control temperature.

We found that the contracture could be eliminated by precooling the tissue at 4°C for 21 to 24 hr prior to use. These tissues were permitted to equilibrate for at least 2 hr at 37.5°C before control dose-response curves were obtained. The precooled tissues were heated in exactly the same manner as described above. With the fresh tissues the minimum time for determining dose-reponse curves after the heating procedure was 2 to 2½ hr, while with the precooled tissues the recovery period varied from 2 to 4 hr.

Mean response values in millimeters for each concentration of control and experimental dose-response curves were calculated. At least 6 experiments were performed with each agonist on each type of tissue (fresh and precooled). The data were analyzed by linear regression analysis, computations being done by an IBM 1620 computer.

Two procedures were used to attempt to ascertain whether angiotensin and serotonin act by releasing ACh. The first entailed the determination of the effect of diisopropyl fluorophosphate (DFP), 1 μg/ml for 10 min, on contractions produced by ACh and by angiotensin. The second involved the effect of *l*-hyoscyamine on contractions elicited by ACh, serotonin, angiotensin, nicotine and tetramethylammonium (TMA). The latter experiments were carried out as follows. After determining complete dose-response curves, *l*-hyoscyamine was applied at a very high concentration (0.1 μg/ml) for 10 min. At this point, the dose-response curve for each drug was redetermined in the presence of the *l*-hyoscyamine; *i.e.*, the buffer solution was made up to contain 0.1 μg/ml of *l*-hyoscyamine.

The drugs used were: acetylcholine chloride (Eastman), serotonin creatinine sulfate (Z. D. Gilman), angiotensin amide (Hypertensin, gift of Ciba Co.), bradykinin (BRS 640, a gift of Sandoz Pharmaceuticals), vasopressin (Pitressin, gift of Parke, Davis & Co.), *l*-hyoscyamine (Parke, Davis & Co.), diisopropyl fluorophosphate (DFP; Aldrich), tetramethylammonium bromide (TMA; K & K Laboratories) and nicotine bitartrate (J. T. Baker Co.).

RESULTS. Heating rat stomach strips to 46°C for periods of time up to 20 min produced somewhat variable results insofar as a selective alteration in the subsequent responses to the various agonists was concerned. Above 47°C, there was a rapid loss in responsiveness to KCl as well as all other contracting agents, indicating destruction of nonreceptor elements of the tissue; 47°C for 20 min seemed to be the best condition for this particular study and all results to be reported (for fresh and precooled tissues) were obtained under these conditions.

ACh responses in fresh and precooled tissues. Figure 1 shows results for the effect of heat on ACh responses in fresh tissues. It is apparent that the heating procedure produced no significant change in the response to either ACh or KCl. The same results were obtained with precooled strips.

Bradykinin responses in fresh and precooled tissues. Figure 2 shows the responses to bradykinin before and after heating fresh tissues. As can be seen, the heating procedure produced no effect on responses to bradykinin. Similar data were obtained with the precooled preparations.

Serotonin responses in fresh tissues. The data in figure 3 are concerned with the effect of heat on responses to serotonin. It is evident that the heat treatment had a profound effect on the responses of the fresh tissue to serotonin, as evidenced by a significant decrease in slope of the experimental dose-response curve. A dose which in the control produced a maximum contraction gave about 25% of this response after heating. It is of interest that, despite marked desensitization to serotonin, a maximum contraction could be elicited. However, the concentration required to produce the maximum response after heating was 1250 times that of the controls.

Serotonin responses in precooled tissues. Unlike the two previous agonists considered, precooling has an effect on the experimental dose-response curve obtained for serotonin. After heat treatment, the serotonin dose-response curve showed a parallel shift to the right. A contraction approximately equivalent to that of the control maximum could be elicited. This required only 50 times the control concentration of serotonin instead of the 1250 times as described for the fresh preparations (fig. 3).

It is of some interest that a distinct difference in the responses of the fresh and

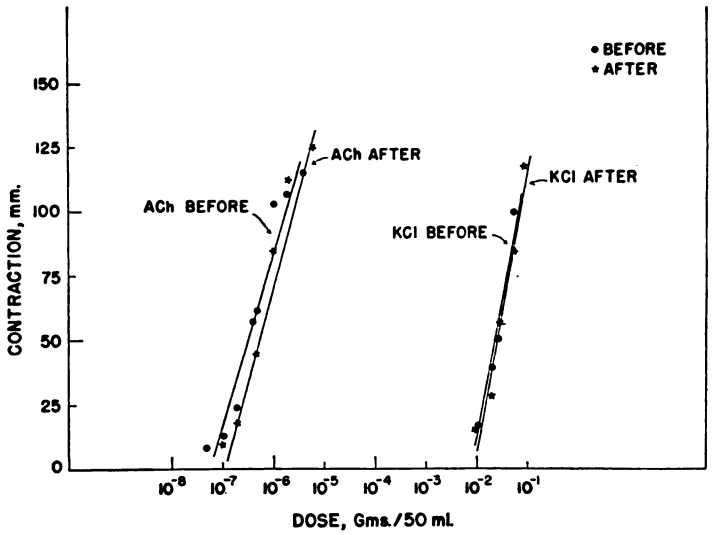

FIG. 1. Effect of heat (47°C, 20 min) on response of rat fundal strip to ACh and KCl; magnification, ×15. Data were plotted according to linear regression analysis. All data refer to responses obtained at 37.5°C. "Before" refers to control dose-response curve; "after" is the dose-response curve after heat treatment and reequilibration of the tissue.

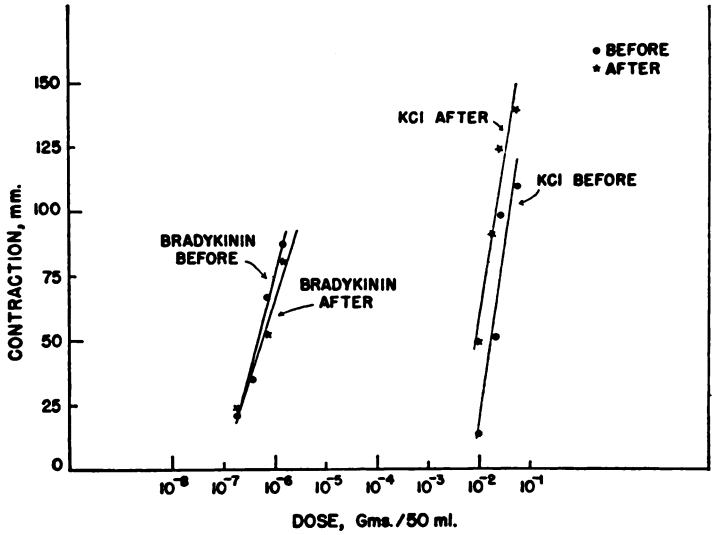

FIG. 2. Effect of heat (47°C, 20 min) on response of rat fundal strip to bradykinin and KCl; magnification, ×15. Data were plotted according to linear regression analysis. All data refer to responses obtained at 37.5°C. "Before" refers to control dose-response curve; "after" is the dose-response curve after heat treatment and reequilibration of the tissue.

precooled tissues to serotonin can be discerned, as shown by comparison of the two control dose-response curves (fig. 3). The slope of the serotonin curve of the precooled preparation was significantly decreased when compared with the controls obtained with the fresh preparation. However, the slopes of the dose-response curves for both preparations were the same after heating.

Angiotensin responses in fresh and precooled tissues. Figure 4 deals with the effect of heat on the response to angiotensin; data for fresh tissues are shown. After heating, responses were found to be greatly depressed. Increasing the

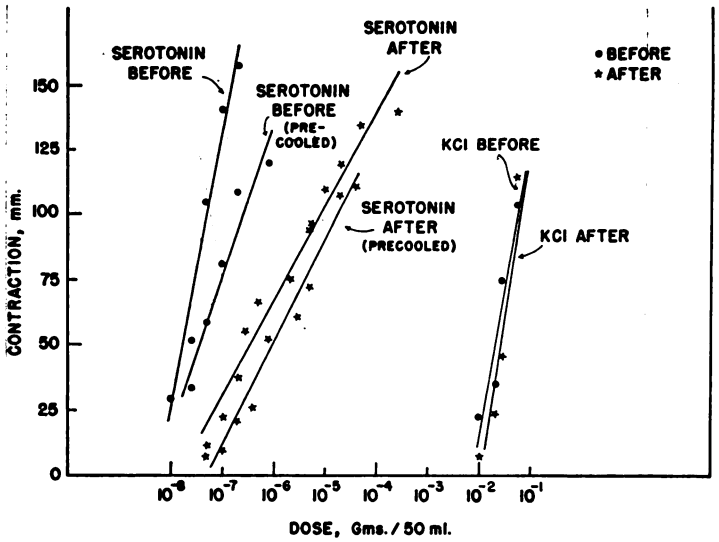

FIG. 3. Effect of heat (47°C, 20 min) on response of rat fundal strip to serotonin and KCl; magnification, ×15. Data were plotted according to linear regression analysis. All data refer to responses obtained at 37.5°C. "Before" refers to control dose-response curve; "after" is the dose-response curve after heat treatment and reequilibration of the tissue.

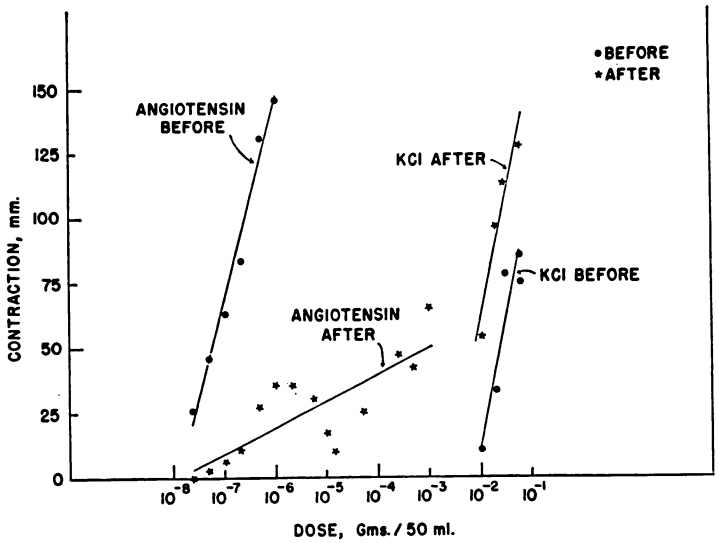

FIG. 4. Effect of heat (47°C, 20 min) on response of rat fundal strip to angiotensin and KCl; magnification, ×15. Data were plotted according to linear regression analysis. All data refer to responses obtained at 37.5°C. "Before" refers to control dose-response curve; "after" is the dose-response curve after heat treatment and reequilibration of the tissue.

concentration of angiotensin even 1000-fold failed to give more than about 50% of the control maximum response. Another type of response was occasionally observed with this agonist after the treatment. The first, second and possibly third doses fell on the control dose-response curve (signifying no decrease in sensitivity) while each subsequent dose produced smaller responses; eventually a response was no longer obtained. This bell-shaped dose-response curve is similar to that seen with indirectly acting sympathomimetic amines (Pruss *et al.*, 1965). However, if the same doses were given once again 1 or 2 hr later, contractions

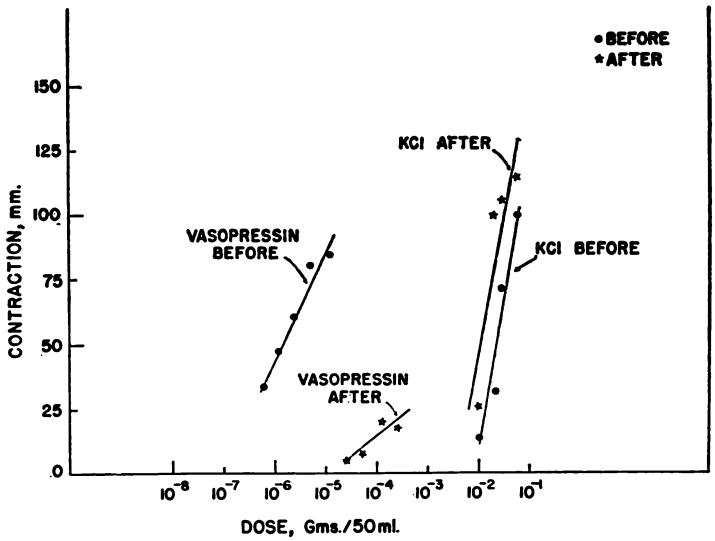

FIG. 5. Effect of heat (47°C, 20 min) on response of rat fundal strip to vasopressin and KCl; magnification, ×15. Data were plotted according to linear regression analysis. All data refer to responses obtained at 37.5°C. "Before" refers to control dose-response curve; "after" is the dose-response curve after heat treatment and reequilibration of the tissue.

were absent although responses to KCl were normal.

Responses to angiotensin using precooled tissues were statistically indistinguishable from those obtained on the fresh tissue.

Vasopressin responses in fresh and precooled tissues. The thermal effect on vasopressin responses was difficult to assess on a fresh tissue, possibly because of catecholamine release by this agent (Gardier *et al.*, 1965). Use of the precooled preparation permitted us to obtain a fairly good dose-respones curve for this polypeptide. This may have been due to a prevention of release of catecholamines in the precooled tissue or to a depletion of stored catecholamines due to reduced synthesis in the cold. As can be appreciated from figure 5, the experimental dose-response curve showed a profound decrease in slope. Only 30% of the maximum contraction height could be obtained even at the highest possible concentration of vasopressin used.[6]

[6] Our results with vasopressin are somewhat clouded by the fact that commercial preparations of the polypeptide contain chlorobutanol, which has recently been shown to be a rather potent smooth muscle depressant (Botting and Manley, 1967). This impurity ordinarily does not significantly affect contractions. After the heat treatment, high concentrations of vasopressin were required to elicit contractions. The amount of chlorobutanol present under these circumstances could conceivably cause tissue depression. Thus, there is some uncertainty as to whether the tissue could be maximally contracted after being heated under the described conditions.

Effect of DFP on responses to angiotensin and ACh. DFP, applied as described, caused a parallel shift in the ACh dose-response curve to the left with an indicated 10-fold potentiation. Under similar conditions, there was no effect on the angiotensin response.

Effect of l-hyoscyamine on responses to ACh, serotonin, angiotensin, TMA and nicotine. *l*-Hyoscyamine, under the conditions used, blocked up to 25 to 100 μg of ACh whereas the maximum contraction was generally achieved at about 800 ng. Nicotine proved to be highly tachyphylactic on this tissue, 1 hr being required between doses. Furthermore, not all strips responded well to this drug. For those which did respond, *l*-hyoscyamine shifted the dose-response curve about 4-fold to the right; in agreement with Vane (1957), control responses were completely abolished by *l*-hyoscyamine. In contrast, TMA responses could be elicited even from those tissues which failed to contract to nicotine. *l*-Hyoscyamine completely blocked concentrations of TMA 10-fold higher than those which gave a maximum contraction in the control. On the other hand, *l*-hyoscyamine had little, if any, effect on dose-

response curves to serotonin or angiotensin. Furthermore, even when the tissue was refractory to nicotine, after a nicotine contraction, responses to both of these agonists were normal.

DISCUSSION. This study represents an attempt to gain insight into the molecular nature of drug receptors situated in smooth muscle by modifying these tissue components *in situ*. The approach used embodies at least one inherent drawback, namely, that this modification is manifested only indirectly through responses of the tissue. The lack of effect on KCl-induced contractions indicated that at least the conducting membrane and contractile elements of the tissue remained intact. The finding that *l*-hyoscyamine and DFP had little, if any, effect on responses to angiotensin and/or serotonin while profoundly altering responses to exogenous or endogenous ACh suggests that these agonists, just like the others examined, contract the tissue by reacting with their own specific receptors. Thus, we feel justified in interpreting any changes in agonist activity in terms of direct receptor modification.

With these considerations in mind, it is necessary to indicate the kinds of information which may be obtained from this type of experiment. Undoubtedly the most heat-labile macromolecules are proteins, although these can have very different thermal stabilities (Putnam, 1953; Scheraga, 1961). Changes in structure, if they occur, are known not to be of the "all or none" type (Putnam, 1953) and thus under certain conditions heat may cause only partial changes in secondary or tertiary structures. Furthermore, there is now well documented evidence that at least some of these changes may be reversed to a greater or lesser extent upon cooling (Putnam, 1953; Scheraga, 1961). Each of these facts must be taken into account when interpreting the data.

The finding that responses to ACh and bradykinin failed to be altered by the heat treatment can be interpreted in one of several ways: 1) the receptors are nonprotein in nature; 2) they are heat-stable proteins; 3) they are proteins which demonstrate fairly rapid reversible denaturation. It is virtually impossible to decide which of these possibilities pertains because of the limitations imposed by the tissue itself, namely, the inability to survive high temperatures for extended periods of time together with the long time-course for tissue recovery. For this reason, we have subjected this tissue to another protein denaturant, urea, with which we were able to demonstrate a distinct effect on responses to these two agonists. These results are discussed elsewhere (Fleisch, 1967; Fleisch and Ehrenpreis, 1968; Ehrenpreis and Fleisch, 1968).

The results with ACh have a distinct bearing on previous suggestions concerning the molecular nature of its receptor. The stability of this receptor to heat is consistent with the concept that cholinesterase can function as an essential part of the ACh receptor apparatus, as suggested by several investigators (see above). This enzyme, whether purified or in intact stomach tissue, is completely stable at 47°C for 20 min (Augustinsson, 1948; Coleman and Eley, 1963; S. Ehrenpreis and A. Chiesa, unpublished observations).

Furthermore, there have been suggestions that the active site on the ACh receptor is comprised of a protein-phospholipid complex (Watkins, 1965; Ehrenpreis, 1967a,b); this too should impart heat stability to this receptor since it is known that lipids protect proteins against thermal denaturation (Boyer *et al.*, 1946). On the other hand, Turpajev *et al.* (1963) reported that the ACh receptor of frog heart was far less stable to heat, being irreversibly inactivated when the tissue was heated to 40°C for 3 to 5 min. It is entirely possible that the receptors as well as the cholinesterase of frog heart have very different thermal stabilities from these components of rat stomach.

There is little doubt that temperature had a profound effect on the response to serotonin. Incubating the tissue in the cold for 21 to 24 hr resulted in a statistically significant reduction in sensitivity to serotonin. Furthermore, both precooled and fresh tissues showed greatly reduced responses to serotonin after the heat treatment, although the control maximum contraction height could be attained. This finding suggests that the serotonin receptor was partially denatured by heat, leading to an altered ability to be activated by its agonist. The effect appeared to be irreversible, since the depressed responsiveness remained for many hours after heating the tissue. Thus, it is concluded that the serotonin receptor is a somewhat heat-labile protein but resists complete destruction when subjected to 47°C for 20 min. Such a conclusion is contrary to the sug-

gestion of Woolley and Gommi (1964) that the active site of the serotonin receptor—or perhaps the entire receptor itself—is composed of a ganglioside, since it would be anticipated that the conformation of such a molecule would not be altered by the comparatively mild treatment employed.

The situation with regard to the angiotensin and vasopressin receptors differed in degree from that of serotonin. It is evident that both of these receptors were profoundly altered by heat, as shown by the greatly diminished responsiveness of the tissue to the polypeptide. Furthermore, the fact that 50% or less of the maximum response could be achieved, regardless of the concentration of agonist used, implies that a definite percentage of the receptors had been completely destroyed by the heat, the remainder being only partially denatured and thus still able to react weakly with their agonists. However, it cannot be stated on the basis of this evidence what fraction of receptors had survived the heating procedure; this may or may not be 50%. The reason for this uncertainty is that there is no information about the number of spare receptors present in this tissue. It is now well recognized that the maximum response is not necessarily brought about by stimulation of the entire population of receptors and indeed may involve only a small fraction of receptors (Furchgott, 1955; Stephenson, 1956; Ariëns *et al.*, 1960).

The effect of heat on the vasopressin receptor is consistent with the suggestion (Schwartz *et al.*, 1960) that a sulfhydryl group is part of the active site on this receptor. Such a group would be found only on a protein molecule; furthermore, according to Cecil (1963), sulfhydryl proteins are readily denatured irreversibly under mild conditions.

This study, in conjunction with the one reported on the action of urea (Fleisch, 1967; Ehrenpreis and Fleisch, 1968; Fleisch and Ehrenpreis, 1968), demonstrates the feasibility of utilizing physical and chemical means to investigate, in a general way, the molecular nature of pharmacologic receptors situated in smooth muscle.

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REFERENCES

ARIËNS, E. J., VAN ROSSUM, J. M. AND KOOPMAN, P. C.: Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs. Arch. Int. Pharmacodyn. Thérap. **127**: 459–478, 1960.

AUGUSTINSSON, K. B.: Cholinesterases, a study in comparative enzymology. Acta Physiol. Scand. **15**: suppl. 52, 1–182, 1948.

BELLEAU, B.: A molecular theory of drug action based on induced conformational perturbation of of receptors. J. Med. Chem. **7**: 776–784, 1964.

BELLEAU, B.: Water as the determinant of thermodynamic transition in the interaction of aliphatic chains with acetylcholinesterase and the cholinergic receptors. *In* Conference on Cholinergic Mechanisms. Ann. N.Y. Acad. Sci. **144**: 705–719, 1967.

BOTTING, J. H. AND MANLEY, D. G.: The action of commercial preparations of oxytocin and vasopressin on the smooth muscle of the gut. J. Pharm. Pharmacol. **19**: 66, 1967.

BOYER, P. D., LUM, F. G., BALLOU, G. A., LUCK, J. M. AND RICE, R. G.: The combination of fatty acids and related compounds with serum albumin. I. Stabilization against heat denaturation. J. Biol. Chem. **162**: 181–198, 1946.

CECIL, R.: The role of sulfur in proteins. *In* The Proteins, ed. by H. Neurath, vol. 1, chap. 5, pp. 379–476, Academic Press, New York, 1963.

CHAGAS, C.: The mechanism of curarization. Ann. N.Y. Acad. Sci. **81**: 345–357, 1959.

COLEMAN, M. H. AND ELEY, D. P.: The thermal inactivation of acetylcholinesterase. Biochim. Biophys. Acta **67**: 646–657, 1963.

DIKSTEIN, S. AND SULMAN, F. G.: Function of phospholipids in receptors. Biochem. Pharmacol. **14**: 739–743, 1965.

EHRENPREIS, S.: Isolation and identification of the acetylcholine receptor protein of electric tissue. Biochim. Biophys. Acta **44**: 561–577, 1960.

EHRENPREIS, S.: Immunohistochemical localization of drug binding protein in tissues of the electric eel. Nature (London) **194**: 586–587, 1962.

EHRENPREIS, S.: Molecular aspects of cholinergic mechanisms. *In* Medicinal Chemistry. I. Drugs Affecting the Peripheral Nervous System, ed. by A. Burger, pp. 1–78, M. Dekker Press, New York, 1967a.

EHRENPREIS, S.: Possible nature of the cholinergic receptor. *In* Conference on Cholinergic Mechanisms. Ann. N.Y. Acad. Sci. **144**: 720–736, 1967b.

EHRENPREIS, S. AND FLEISCH, J. H.: Alteration in vasoactive polypeptide receptor activity of rat fundal strip by heat and urea. Federation Proc. **27**: 70–72, 1968.

EHRENPREIS, S., HAZRA, J. AND BIGO-GULLINO, M.: Effects of acetone on responses of a number of tissues to biogenic amines. Biochem. Pharmacol., in press, 1968.

FLEISCH, J. H.: Inactivation of smooth muscle receptors by urea. Federation Proc. **26**: 736. 1967.

FLEISCH, J. H. AND EHRENPREIS, S.: A study of the alteration of smooth muscle receptors by urea. J. Pharmacol. Exp. Therap., in press, 1968.

FURCHGOTT, R. F.: The pharmacology of vascular smooth muscle. Pharmacol. Rev. **7**: 183–266, 1965.

GADDUM, J. H. AND PICARELLI, Z. P.: Two kinds of tryptamine receptors. Brit. J. Pharmacol. Chemotherap. **12**: 323–328, 1957.

GARDIER, R. W., RICHARDS, A. B., JAMES, E. A., JR. AND WHEELER, J. E.: Vasopressin vasodynamics. I. The pharmacology of tachyphylaxis. Arch. Int. Pharmacodyn. Thérap. **153**: 232–239, 1965.

HASSON, A., AND CHAGAS, C.: Purification of macromolecular components of the aqueous extract of the electric organ (E. Electricus) with binding capacity in vitro for quaternary ammonium bases. *In* Bioelectrogenesis, ed. by C. Chagas and A. P. de Carvalho, pp. 362–378, Elsevier Publishing Co., Amsterdam, 1961.

KHAIRALLAH, P. A. AND PAGE, I. H.: Mechanism of action of angiotensin and bradykinin on smooth muscle in situ. Amer. J. Physiol. **200**: 51–54, 1961.

OFFERMEIER, J. AND ARIENS, E. J.: Serotonin 1. Receptors involved in its action. Arch. Int. Pharmacodyn. Thérap. **164**: 192–215, 1966.

PRUSS, T. P., MAENGWYN-DAVIES, G. D. AND WURZEL, M.: Comparison of effects of aromatic sympathomimetic amines on rabbit aortic strip and rabbit blood pressure. J. Pharmacol. Exp. Therap. **147**: 76–85, 1965.

PUTNAM, F. W.: Protein denaturation. *In* The Proteins. ed. by H. Neurath and K. Bailey, vol. I, part B, chap. 9, pp. 807–892, Academic Press, New York, 1953.

SCHERAGA, A.: Protein Structure, chap. IV, Academic Press, New York, 1961.

SCHWARTZ, I. L., RASMUSSEN, H., SCHOESSLER, M. A., SILVER, L., AND FONG, C. T. O.: Relation of chemical attachment to physiological action of vasopressin. Proc. Nat. Acad. Sci. USA **46**: 1288–1298, 1960.

STEPHENSON, R. P.: A modification of receptor theory. Brit. J. Pharmacol. Chemotherap. **11**: 379–393, 1956.

TURPAJEV, T. N., NISTRATOVA, S. N. AND PUTINTSEVA, T. G.: Protein nature of acetylcholine receptor and the role of energy rich compounds in the realization of acetylcholine action. *In* Proceedings of the First International Pharmacological Meeting, Stockholm, vol. VII, Modern Concepts in the Relationship between Structure and Pharmacological Activity, ed. by K. J. Brunings, pp. 145–155, Pergamon Press, New York, 1963.

UMBRIET, W. W., BURRIS, R. H. AND STAUFFER, J.: Manometric Techniques and Tissue Metabolism, p. 149, Burgess Publishing Company, Minneapolis, 1957.

VANE, J. R.: A sensitive method for the assay of 5-hydroxytryptamine. Brit. J. Pharmacol. Chemotherap. **12**: 344–349, 1957.

WATKINS, J. C.: Pharmacological receptors and general permeability phenomena of cell membranes. J. Theor. Biol. **9**: 37–50, 1965.

WOOLLEY, D. W.: Serotonin receptors. I. Extraction and assay of a substance which renders serotonin fat soluble. Proc. Nat. Acad. Sci. USA **44**: 1202–1210, 1958.

WOOLLEY, D. W.: Extraction and assay in vitro of an "acetylcholine receptor (?)." Federation Proc. **18**: 461, 1959.

WOOLLEY, D. W. AND GOMMI, B. W.: Serotonin receptors. V: Selective destruction by neuraminidase + EDTA and reactivation with tissue lipids. Nature (London) **202**: 1074–1075, 1964.

WOOLLEY, D. W. AND GOMMI, B. W.: Serotonin receptors. VI. Methods for direct measurement of isolated receptors. Arch. Int. Pharmacodyn. Thérap. **159**: 8–17, 1966.

WURZEL, M.: The physiological role of cholinesterase at cholinergic receptor sites. *In* Conference on Cholinergic Mechanisms. Ann. N.Y. Acad. Sci. **144**: 694–704, 1967.

ŽUPANČIČ, A. O.: Mode of action of acetylcholine. Acta Physiol. Scand. **29**: 63–71, 1953.

ŽUPANČIČ, A. O.: Evidence for the identity of anionic centers of cholinesterases with cholinoreceptors. *In* Conference on Cholinergic Mechanisms. Ann. N.Y. Acad. Sci. **144**: 689–693, 1967.