

## SECOND MESSENGERS: RATE OF FORMATION AS AN INDEX OF RECEPTOR REACTIVITY

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*Abstract.* Generation of a second messenger upon stimulation of a metabotropic membrane receptor is the first biochemical reaction in the process of translation of a signal approaching the cell into the cellular response. Thus, the measurement of the rate of generation of these intracellular chemical signals may be a convenient way to assess the functional state of the receptor. The basic methodologies for assessment of the formation of cyclic AMP and inositol phosphates are described. Examples from our laboratory show how the measurement of these messengers may be employed in studies on receptor adaptation to permanently changed neurotransmitter availability, the discrepancy between the changes of receptor densities and functional up- or down-regulation, cooperativity of receptors, mechanisms of the action of drugs, neurotransmission changes in senescence and comparative neurochemistry.

### INTRODUCTION

The concept of receptor was formulated to explain the results of *in vitro* studies on isolated organs, and the size of contractile response was regarded as a measure of receptor reactivity (3, 16). With the broadening

of the concept of receptor and finding out that several reactions are mediated through interaction with these specific membrane domains, the need for a general method of assessment of receptor reactivity became apparent. It has been postulated that the size of responses depends on two independent receptor parameters: the affinity and intrinsic activity (2), and the definition of the latter entity posed some problems. The introduction of the methodology of radioligand binding (see e.g. 91, 92) aroused a considerable interest, as reflected by a plethora of studies, in which the density and affinity of receptors were reported. It was generally believed that changes in those characteristics reflect the changes in the functional status of the receptor. However, the radioligand binding assay characterizes only the recognition unit of a receptor, while the receptor response, triggered by stimulation of the recognition unit, involves several postrecognition biochemical events, leading to the final effect of receptor stimulation, namely the change in the postsynaptic cell.

The receptors may be broadly classified in two large categories: ionotropic and metabotropic ones (see 85). In the latter, the postsynaptic change resulting from receptor stimulation does not occur directly, but involves a second messenger: a low-molecular species generated upon stimulation of the receptor at the cell membrane and acting directly within the postsynaptic cell. The number of substances which were found to play the role of a second messenger increases. In this article we shall confine ourselves to two, probably the best characterized, second messenger systems: those of cyclic AMP and phosphatidylinositols. They are linked with various receptors either in a stimulatory or inhibitory fashion (5, 6, 69). For the cyclic AMP system the possibility of stimulatory or inhibitory connection arises from the fact that the second messenger generating system is not connected with a receptor directly, but through an intermediary link, a protein known as G protein (11, 32, 33, 62 - 64, 68). There are several types of G proteins: some of them, upon receptor stimulation, activate enzymatic systems synthesizing second messengers, other inhibit them (52, 53, 90). A G protein is composed of three subunits,  $\alpha$ ,  $\beta$  and  $\gamma$  (63). While  $\beta$  and  $\gamma$  subunits are very similar if not identical in all G proteins, the  $\alpha$  subunits are different and they determine the character of a G protein: those having stimulatory  $\alpha_s$  subunit become stimulatory  $G_s$  proteins, while those having inhibitory  $\alpha_i$  subunit are inhibitory  $G_i$  proteins. There exist also other G proteins, such as  $G_o$ ,  $G_t$ , etc. However, the description of G proteins is definitely beyond the scope of this review. For the phosphatidylinositol system stimulatory or inhibitory receptor links are also described, but the molecular mechanisms underlying them are still little known.

In our laboratory we employ the measurement of the rate of formation of cyclic AMP and inositol phosphate to assess the functional status and responsiveness of receptors and their change in various conditions. The main assumption of the approach, consisting of the assessment of second messenger formation for this purpose, is that this response is the first biochemical reaction following receptor stimulation, and therefore is crucial for the following chain of reactions. The ultimate response, whether a contraction of a muscle, secretion of a hormone, or change in behavior, may, of course, be modified, nullified or enhanced by several processes acting "downstream" the receptor. Therefore, the ultimate response, or any other measurable response below the stage of formation of a second messenger, may not reflect precisely the reactivity of receptor, which is crucial for the effectiveness of neurotransmission.

## THE CYCLIC AMP SYSTEM

### *Theory*

The cyclic AMP system is linked to such receptors as  $\beta$ - and  $\alpha_2$ -adrenoceptors, serotonin 5HT<sub>1A</sub> and 5HT<sub>1B</sub>, dopamine D<sub>1</sub> and histamine H<sub>2</sub> receptors (17, 18, 21, 37, 59, 77). The  $\beta$ -adrenoceptor is a classic in this respect, as the hypothesis of the existence of second messengers evolved from Earl Sutherland's work on the involvement of adrenoceptors in glycolysis (79). Moreover, the  $\beta$ -adrenoceptor became a target of psychopharmacological studies, and the assessment of the functional state of this receptor by the measurement of cyclic AMP accumulation upon noradrenaline challenge, carried out by Vetulani and Sulser in 1975 (10, 85 - 87), revealed the downregulation of  $\beta$ -adrenoceptor in the course of treatment with antidepressant drugs. Currently many laboratories, including ours, are still involved in the assay of cyclic AMP to measure the  $\beta$ -adrenoceptor responsiveness to noradrenaline and more specific agonists.

A schematic picture of the mechanisms involved in the formation of cyclic AMP is presented in Fig. 1. The stimulation of a  $\beta$ -adrenoceptor, which is linked to a stimulatory protein G<sub>s</sub>, leads to activation of adenylate cyclase and, in turn, an increased generation of cyclic AMP from ATP. The second messenger thus formed reacts with protein kinase A and activates it. Activated kinase A phosphorylates specific cellular proteins. Phosphorylation of proteins changes their conformation, and this change leads to a postsynaptic response to the external signal received by the receptor. The cyclic AMP is degraded by cyclic AMP phosphodiesterase and converted to noncyclic adenosine monophosphate. If a G protein is of an inhibitory type (G<sub>i</sub>), as e.g. in case of  $\alpha_2$ -adrenoceptor, the

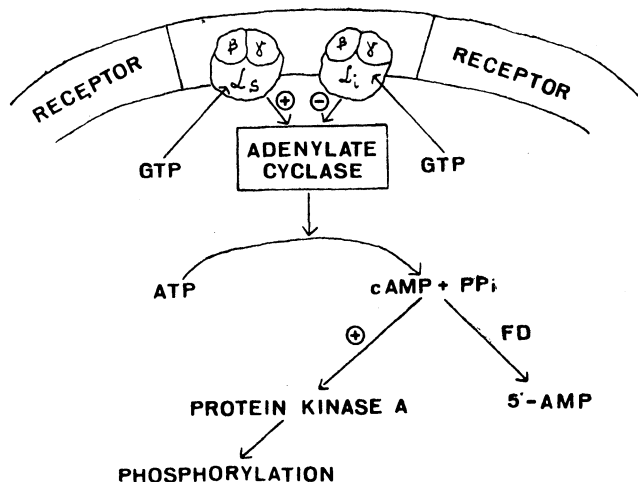


Fig. 1. The schematic picture of the mechanisms involved in formation of cyclic AMP. The picture presents the membrane receptors linked with G proteins, composed of three subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ . Depending on the  $\alpha$  subunit ( $\alpha_s$  or  $\alpha_i$ ), the G protein may be either stimulatory ( $G_s$ ) or inhibitory ( $G_i$ ). Upon connection with GTP the G protein activates ( $G_s$ ) or inhibits ( $G_i$ ) adenylate cyclase which converts ATP into cyclic AMP (cAMP). The latter activates protein kinase A, which, in turn, phosphorylates various cell proteins. Cyclic AMP is destroyed by phosphodiesterase (FD) to form 5'-AMP.

stimulation of such a receptor results in the inhibition of cyclic AMP formation.

### Methodology

As the assay of cyclic AMP is carried out by many research groups, the standardization of the method of its assessment is a crucial issue. An assay of cyclic AMP as a measure for activation of  $\beta$ -adrenoceptor is not simple in an *in vivo* situation. Because of different oxygen requirements for the synthesizing enzyme — adenylate cyclase, and the degrading enzyme — cyclic AMP phosphodiesterase, a rapid postmortem increase in cyclic AMP completely obliterates more subtle changes (43). If no proper measures are taken — and the only sufficient for mice is microwaving the animals, and for rats — microwaving with a specially focused beam, allowing the core brain temperature to increase to approx,  $70^\circ\text{C}$  within 1-2 seconds (74) or microwaving immediately the decapitated head (22), the results are meaningless. The freeze-blowing technique is an alternative, but makes impossible studies in brain regions (44, 83). Dropping animals into liquid nitrogen is not enough (15).

Because of these difficulties, most of the work is carried out on cerebral slices. The crucial issue in these experiments is maintaining the slices in proper conditions. If oxygenation is not sufficient and the equilibration time too short, the basal results will be erroneously high, and the results worthless. The basal level of cyclic AMP is a good index of proper experimental conditions.

There are two basic methodologies of cyclic AMP assay: the protein binding assay method based on displacement of labeled cyclic AMP from protein kinase by cold cyclic AMP extracted from the sample (12, 29) (most commercial kits for cyclic AMP assay are based on this methodology), and a more dynamic method of assessment of cyclic AMP formation, based on the measurement of the percentage of conversion to cyclic AMP of radioactive ATP, formed from tritiated adenine added to the incubation mixture. The method which is used in our laboratory, based on that of Shimizu (71), belongs to the second group.

The basic steps of the method are as follows:

*Preparation of tissue.* The brain of a decapitated animal is rapidly removed from the skull and placed on an ice-chilled glass or porcelain plate. The required brain region is excised and slices from this tissue are prepared. The best results are obtained using a McIlwain tissue chopper, set for 300  $\mu\text{m}$  and cutting the tissue in two perpendicular directions. The slices thus obtained are thin enough to secure sufficient diffusion of oxygen and glucose into the cell and large enough to be treated as an unbroken cell preparation.

*Preincubation — adaptation.* The slices are suspended in  $\text{O}_2 : \text{CO}_2$  (95 : 5) gassed, glucose-containing modified Krebs-Henseleit medium (NaCl, 118 mM, KCl, 5 mM;  $\text{CaCl}_2$ , 1,3 mM;  $\text{MgSO}_4$ , 1,2 mM;  $\text{KH}_2\text{PO}_4$ , 1,2 mM;  $\text{NaHCO}_3$ , 25 mM; glucose 11,7 mM; pH 7,4) at 37°C for a 15 min adaptation period.

*Labeling incubation.* The buffer is changed and [ $^3\text{H}$ ]adenine is added to the incubation mixture in final conc. of 100 nM. The incubation is continued for 45 min.

*Incubation with agonists.* The slices are washed, density-packed and distributed in 50  $\mu\text{l}$  portions into vials containing 440  $\mu\text{l}$  of the buffer. After 5 min of preincubation, the agonist (10  $\mu\text{l}$ ) and tested drugs (10  $\mu\text{l}$ ) are added and the slices are incubated for 10 min.

*Stopping incubation.* The reaction is stopped with 550  $\mu\text{l}$  of 10% trichloroacetic acid, the mixture is homogenized and centrifuged, and the supernatant is decanted into test tubes.

*Purification of formed [ $^3\text{H}$ ]cyclic AMP.* An aliquot of 50  $\mu\text{l}$  of the supernatant is taken for assay of total radioactivity. To the remaining supernatant a solution of [ $^{14}\text{C}$ ]cyclic AMP is added (tracer for calculation

of recovery) and the mixture is passed through a system of two columns (Dowex 50 W  $\times$  4 and alumina) placed one above the other. The column system is eluted with water and then the alumina column is eluted with 0.1 M imidazole solution.

*Radioactivity counting.* The final eluate is tested for radioactivity in a liquid scintillation counter ( $[^{14}\text{C}]/[^3\text{H}]$  channel).

## THE PHOSPHATIDYLINOSITOL SYSTEM

### *Theory*

The phosphatidylinositol system was discovered as a second messenger system much later than the cyclic AMP system. The first hints of it were given by Hokins in 1955 (36), and then the hypothesis was elaborated by Berridge (5, 6, 8). The system employs membrane phosphatidylinositols, which are hydrolyzed to inositol phosphates and diacylglycerols by phospholipase C activated by stimulation of such receptors as  $\alpha_1$ -adrenergic, serotonergic 5HT<sub>2</sub> and 5HT<sub>1C</sub>, histamine H<sub>1</sub>, muscarinic M<sub>1</sub> and various peptidergic receptors (1, 7, 13, 34, 35, 40, 46).

The formation of various second messengers in the two main pathways of phosphatidylinositol metabolism is presented in Fig. 2.

The phosphatidylinositol system is present in cell membranes and consists of three forms of phosphatidylinositols increasing in the degree of phosphorylation: phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>). After the activation of a receptor linked in a stimulatory manner with phosphatidylinositol system (most known receptors in this system are linked in a stimulatory manner), the activation of an appropriate G protein results in an activation of phospholipase C, an enzyme catalyzing PIP<sub>2</sub> hydrolysis (65). This results in the formation of various diacylglycerols (DG) and inositol trisphosphate (IP<sub>3</sub>). The assay of DG is carried out using [<sup>3</sup>H]glycerol followed by TLC separation, but this method is not specific enough to assess the DG formed in the brain after phospholipase C activation (48). Most of authors, including us, use only an assay of the second messengers of inositol phosphate group. Studies with DG analogues as well as measurement of the effects of activation of receptors of the phosphatidylinositol system not explainable by the effects of IP<sub>3</sub> indicate that DG activates protein kinase C (47), an agent phosphorylating most of intracellular proteins (49, 50, 78). A discussion of the further effects of protein kinase C activation is beyond the scope of this article.

The first active compound in the chain of inositol phosphates is IP<sub>3</sub>, which acts by releasing calcium from the endoplasmic reticulum (1, 24,

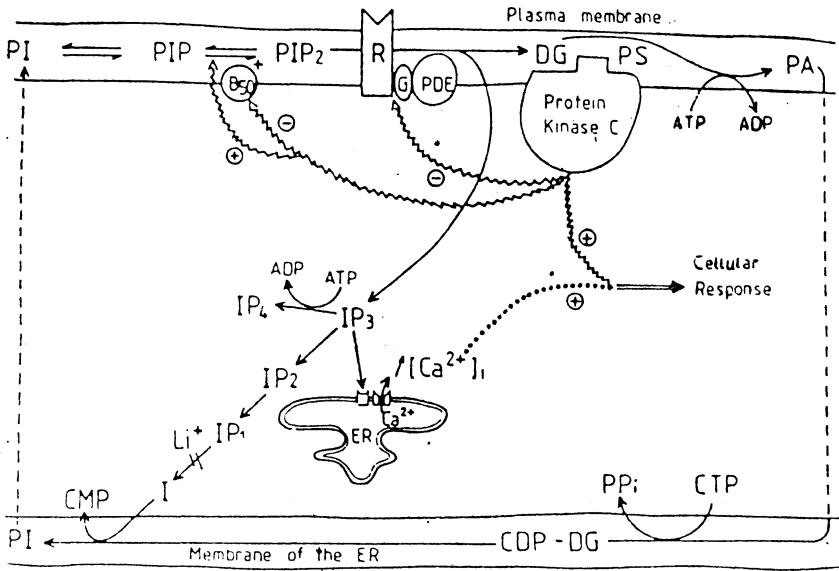


Fig. 2. Phosphoinositide cycle and schematic representation of IP<sub>3</sub>/Ca<sup>2+</sup> and DG/kinase C pathways (From 71). PI is transferred from endoplasmic reticulum (ER) membrane to cellular membrane where it is phosphorylated; the phosphoinositides of various degree of phosphorylation (PI, PIP, PIP<sub>2</sub>) are in equilibrium. Upon stimulation of receptor R the appropriate G protein (G) activates phosphodiesterase of phosphoinositides (phospholipase C) (PDE), which cleaves PIP<sub>2</sub> into diacylglycerol DG and inositol trisphosphate IP<sub>3</sub>. DG activates protein kinase C, which for full activity requires also the presence of phosphatidyl serine (PS). Protein kinase C phosphorylates several cellular proteins, including the brain-specific B<sub>50</sub> protein, the receptor protein and, in the presence of cytoplasmic Ca<sup>2+</sup> affects several other cellular reactions. DG, may form phosphatidic acid PA, which is transferred into the ER membrane, where reacting with cytosine trisphosphate CTP forms CDP-DG. IP<sub>3</sub>, which acts by releasing Ca<sup>2+</sup> from ER, may be either phosphorylated further to form IP<sub>4</sub> (which acts as a calcium ionophore) or is dephosphorylated to IP<sub>2</sub> and further on to IP and, finally, to inositol (I). Inositol enters the ER membrane where it reacts with the CDP-DG. The formed PI reenters the cellular membrane.

34). This leads to an increase in the intracellular calcium concentration. Calcium, by the way, is also regarded as a second messenger, as it activates several calcium-dependent proteins, particularly calmodulin and — via this mechanism — calmodulin-dependent kinases (5, 6, 8). IP<sub>3</sub> may undergo phosphorylation or dephosphorylation. Phosphorylation of IP<sub>3</sub> leads to the formation of inositol tetrakisphosphate, IP<sub>4</sub>, also a second messenger, which is probably responsible for an increase in the calcium influx to the cell (9, 31, 39, 66, 81). The dephosphorylation products, IP<sub>2</sub>, IP and dephosphorylated inositol are not active as second messengers (9, 73); the reaction chain leads to incorporation of the last

substance into the phosphatidylinositol system. This chain of dephosphorylations may be inhibited at the IP stage by adding lithium ions (20, 23). The addition of lithium results in an accumulation of IP, which might be measured, and its accumulation may serve as an index of second messenger generation by the phosphatidylinositol system. However, caution is needed, as DG can be formed not only from PIP<sub>2</sub>, but also from less phosphorylated phosphatidylinositols (5, 6, 38), and as yet no direct method of assay of this activity is available. Besides, an assay of IP does not inform us how much IP<sub>4</sub> has been formed. Apparently, the assay of all inositol phosphates separately is the best mode of analysis of second messengers in this system. This may be carried out using HPLC methods (23, 80, 82). Recently Amersham introduced a kit for IP<sub>3</sub> assay. However, presently still most of the researchers assay IP after inhibition of its degradation with lithium. It should always be remembered, however, that with such a method a part of the story is still unknown.

The most investigated, because of the large response, were the systems related to  $\alpha_1$ -adrenergic and muscarinic M<sub>1</sub> receptors (25 - 27, 41, 42, 45). In our Institute we used the IP assay to find out whether the changes found in the density of  $\alpha_1$ -adrenergic receptors in the course of antidepressant treatment are significant functionally. With this method we also investigated, in cooperation with the Istituto Superiore di Sanità in Rome, the changes in  $\alpha_1$ -adrenergic and M<sub>1</sub> muscarinic receptors in senescence.

### *Methodology*

The most popular methods of assessment of the activity of phosphatidylinositol system employ tissue slices and consist in the assay of inositol phosphate (IP) formed during hydrolysis of phosphatidylinositols. The methodology used for brain tissue is essentially based on that of Brown et al. (13), using LiCl in a final conc. of 5 mM to inhibit IP degradation.

The incubation of slices proceeds in three stages:

*Preincubation.* The slices obtained from a brain region of a single rat (vide supra) are incubated in oxygenated, glucose-containing modified Krebs-Henseleit medium (the same as that used for cyclic AMP assay) for 1 h, with three changes of the buffer. This stage serves to regenerate the energy in the cell.

*Labeling incubation.* Portions of 50  $\mu$ l of preincubated, density-packed slices are pipetted into flat-bottom 3 ml plastic vials containing 200  $\mu$ l of the buffer, 30  $\mu$ l of LiCl solution (final conc. 5 mM) and 20  $\mu$ l of the purified [<sup>3</sup>H]myoinositol solution (S.A. 19 - 20 Ci/mmol). The vials are gassed (95% O<sub>2</sub> : 5% CO<sub>2</sub>), capped, and shaken at 37°C for 30 min. During

this stage [ $^3\text{H}$ ]myoinositol is being incorporated into the membrane phospholipids, forming phosphatidylinositols (PI, PIP, PIP<sub>2</sub>).

*Hydrolytic incubation.* The incubation starts with an addition of 10  $\mu\text{l}$  of an agonist solution to the medium. At this stage the agonists, acting via appropriate receptors, activate phospholipase C, which catalyzes the hydrolysis of PIP<sub>2</sub> with liberation of inositol phosphates. The incubation continues for 45 min and is stopped by adding of 1,000  $\mu\text{l}$  of a chloroform-methanol (1 : 2) mixture. To separate the phases, a further 350  $\mu\text{l}$  of chloroform and 350  $\mu\text{l}$  of water are added and the mixture is centrifuged for 10 min at 1,000 g. A portion of 1,000  $\mu\text{l}$  of the upper aqueous phase is transferred into vials containing 2.5 ml of water and 0.5 ml of 50% (w/v) slurry of Dowex to bind the phosphates. After 3 washings with 5 ml portions of 5 mM aqueous myoinositol solution, the phosphates are eluted with 600  $\mu\text{l}$  of a mixture of 1 M ammonium formate and 0.1 M formic acid (1 : 1). A portion of 500  $\mu\text{l}$  of this eluate is added to 3 ml of Bray's fluid and counted for radioactivity in a liquid scintillation counter.

#### SOME APPLICATIONS OF SECOND MESSENGER ASSAY IN NEUROSCIENCES

The assay of second messengers has found wide applications in neurosciences. We shall discuss only a few of those which were used in experiments carried out by us.

##### *Adaptive receptor changes*

In mid-'70s Vetulani and Sulser applied cyclic AMP assay to assess the functional state of adrenoceptors in rats treated chronically with antidepressants and electroshock (87 - 89). Chronic administration of antidepressants — desipramine and iprindol, reduced the accumulation of cyclic AMP in brain slices of rats challenged with noradrenaline; the degree of inhibition was independent of the actual drug concentration, and therefore the effect was regarded as an adaptive process resulting from a change in the receptors (87). A depression of cyclic AMP accumulation after noradrenaline was observed also in preparations from rats receiving chronically monoamine oxidase inhibitors, nialamide and pargyline. No such effects were observed after a single or short-term administration of the drugs (88). In addition, depletion of noradrenaline from the brain by reserpization or central chemosympathectomy with 6-hydroxydopamine resulted in an increased responsiveness of the cyclic AMP generating system to noradrenaline (85, 87). These results were the basis for the presently still very popular hypothesis of etiology of depression and the mechanism of action of antidepressant drugs.

*Similarities and discrepancies between changes in receptor density and second messenger response*

Several studies have demonstrated that the decreased responsiveness of the cyclic AMP generating system after chronic antidepressant treatment is paralleled by the decline in density of  $\beta$ -adrenoceptors (see (86)). However, our studies suggest that there is not always a parallelism between the changes in receptor density and response measured by second messenger assay. Thus, we reported (84) that after chronic antidepressant treatments of various kinds the  $\alpha_1$ -adrenergic receptor density increases. On the other hand, no changes in IP accumulation were observed by us (60) and by others (51) after chronic ECS or imipramine treatment. Other authors reported either increases (57) or decreases (75).

*Characteristics of receptors involved in the second messenger response*

It has been generally assumed that the increase in cyclic AMP accumulation in slices treated with noradrenaline results from the stimulation of  $\beta$ -adrenoceptors. However, a specific  $\beta$ -adrenoceptor agonist, isoproterenol, produces a much weaker effect in cerebral slices (72). This suggests that another receptor system is also involved in the overall response to noradrenaline, which is an agonist of all types of adrenergic receptors (70, 76). Our studies (60) indicate that if isoproterenol is added to the incubation medium together with a specific  $\alpha$ -adrenergic agonist, 6-fluoronoradrenaline, the response of the cyclic AMP is close to that induced by noradrenaline. This suggests cooperation between cerebral  $\alpha_1$ - and  $\beta$ -adrenoceptors in the generation of cyclic AMP.

*Detailed studies on the mechanism of adaptive changes produced by antidepressant treatments*

If the cyclic AMP response depends in part on coupling between  $\alpha_1$ - and  $\beta$ -adrenoceptors, the question arises whether chronic antidepressant treatment changes the responses linked to the  $\beta$ -adrenoceptor alone or to the coupling. Our studies suggest that the changes in coupling may be responsible, at least in part, for the so-called  $\beta$ -downregulation. We have found (60) that after chronic electroshock the cyclic AMP response to noradrenaline is inhibited, while the response to isoproterenol is affected only slightly, and in this case the effect did not reach the level of significance. However, the response to combined treatment with isoproterenol and 6-fluoronoradrenaline is strongly attenuated (Fig. 3). A similar experiment on cortical slices of rats treated chronically with imipramine shows a similar pattern, though in this case the inhibition of the

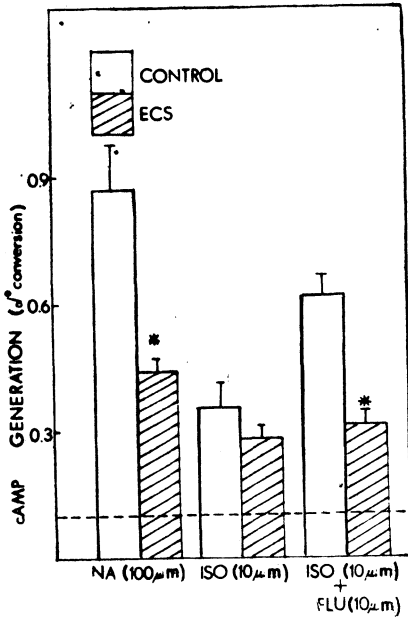


Fig. 3. The effect of chronic electroconvulsive treatment on cyclic AMP generation stimulated by noradrenaline (NA), isoproterenol (ISO) and 6-fluoronoradrenaline (6FLU).  $n = 5-6$ . The broken line shows basal level. Two important points should be noted: 1. addition of 6-fluoronoradrenaline, an  $\alpha$ -adrenoceptor agonist which by itself does not stimulate cyclic AMP generation, markedly increases the effect of isoproterenol, a specific  $\beta$ -adrenoceptor agonist, which is much less effective in stimulation cyclic AMP formation than noradrenaline, a mixed  $\alpha$  and  $\beta$ -adrenoceptor agonist. 2. Chronic electroconvulsive treatment, which significantly attenuated the stimulatory effect of noradrenaline (to the level similar to that observed after isoproterenol stimulation, does not affect the stimulatory effect of isoproterenol. However, it abolishes the 6-fluoronoradrenaline-induced potentiation of the effect of isoproterenol. From (14).

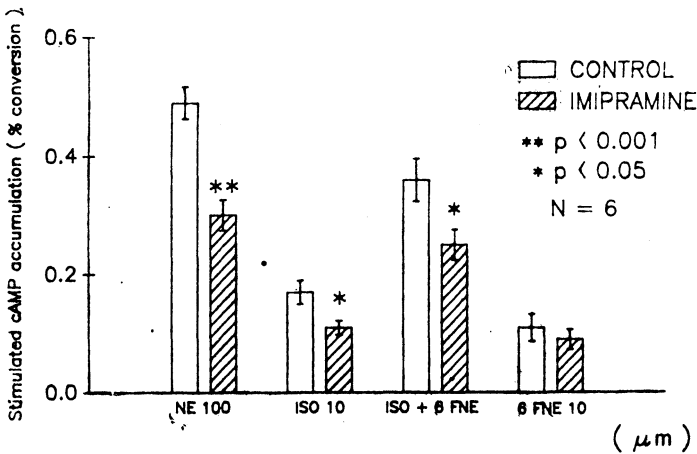


Fig. 4. The effect of chronic imipramine treatment on cyclic AMP generation stimulated by noradrenaline (NE), isoproterenol (ISO) and 6-fluoronoradrenaline (6 FNE). Noradrenaline was given in conc. of 100  $\mu\text{M}$ , isoproterenol and 6-fluoronoradrenaline — 10  $\mu\text{M}$ . The ordinate presents the conversion rate of adenine into cyclic AMP after subtraction of the basal conversion rate. In contrast to the experiment on the effect of electroconvulsive treatment, chronic administration of imipramine attenuates also the isoproterenol-induced stimulation of cyclic AMP formation. However, the treatment abolishes the 6-fluoronoradrenaline-induced potentiation of the effect of isoproterenol. From (60).

effect of isoproterenol reached the level of significance (Fig. 4). The results suggest that the  $\alpha$ - $\beta$ -adrenoceptor coupling may be one, but not the only, target of the adaptive changes produced by antidepressant treatment.

#### *Studies on changes in receptor responsiveness in senescence*

It is generally believed that important changes in neurotransmitter systems appear in senescence (30). However, in Wistar and Sprague Dawley rats no changes in the density of  $\alpha_1$ -adrenergic and muscarinic receptors in the cerebral cortex were noted in senescence (54, 61, 67). We assessed, therefore, the responsiveness of cerebral cortical  $\alpha_1$ -adrenoceptors and cholinergic muscarinic  $M_1$  receptors in adult (3 months old) and senescent (24 months old) male Sprague-Dawley rats by assay of inositol phosphate (IP) formation in cortical slices stimulated by nor-

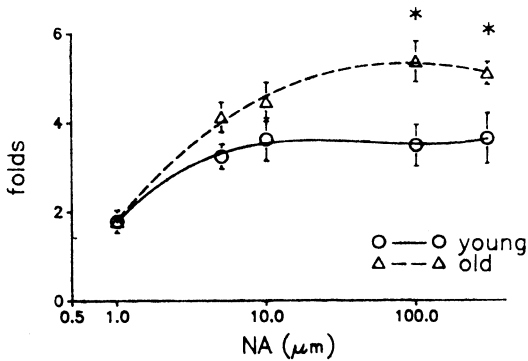


Fig. 5. Higher responsiveness of  $\alpha_1$ -receptor system in old than in young rats, as demonstrated by the accumulation of inositol phosphate (IP) in cerebral slices after stimulation with noradrenaline. Ordinate: the IP concentrations (as multiplicity of the basal level). Abscissa: the concentration of noradrenaline (NA). The points represent means  $\pm$  SEM from 6 experiments. Asterisks denote a significant difference between old and young subjects. From (56).

adrenaline and carbachol (56). The responsiveness of the phosphatidylinositol system to a challenge with noradrenaline was greater in old rats (Fig. 5). A similar result was obtained with slices challenged with carbachol (Fig. 6). This suggests a greater responsiveness of  $\alpha_1$ -adrenoceptors and cholinergic muscarinic  $M_1$  receptors in aged rats. Interestingly, it seems that the increase in the responsiveness of muscarinic  $M_1$  receptors has a behavioral correlate: old Wistar rats are more responsive to the action of a muscarinic receptor agonist, oxotremorine (58). This study is another example demonstrating that in some cases the examination of second messenger systems may be more relevant than receptor binding

studies. It seems that the increased responsiveness of the phosphoinositide system in old age may be regarded as a compensatory mechanism. In old age the cellular calcium homeostasis is impaired (4), the brain calcium uptake is reduced and the calcium availability for the cell is decreased (28). Phosphoinositide hydrolysis may be important for chang-

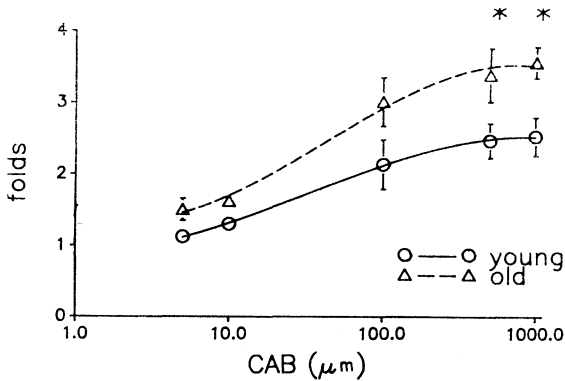


Fig. 6. Higher responsiveness of cholinergic M<sub>1</sub> receptor system in old than in young rats, as demonstrated by the accumulation of inositol phosphate (IP) in cerebral slices after stimulation with noradrenaline. Ordinate: the IP concentrations (as multiplicity of the basal level). Abscissa: the concentration of carbachol (CAB). The points represent means  $\pm$  SEM from 6 experiments. Asterisks denote a significant difference between old and young subjects. From (56).

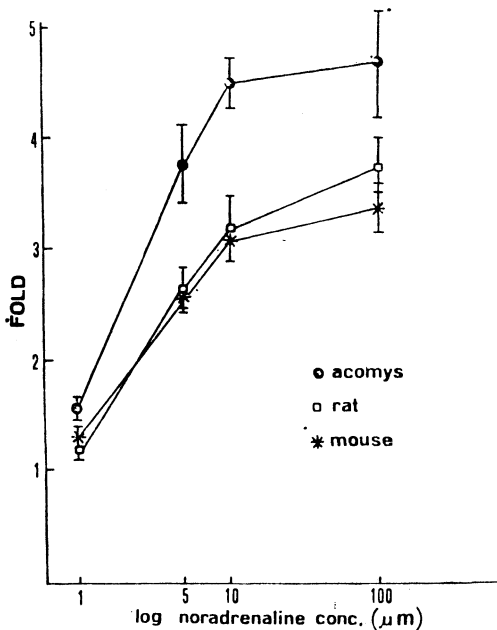


Fig. 7. The differences in noradrenaline effect on IP accumulation in the cerebral cortical slices of *Acomys cahirinus*, Wistar rat and Swiss albino mouse. Ordinate: IP concentration (as multiplicity of the basal level), abscissa — noradrenaline concentration. The points represent the means  $\pm$  SEM of 10 (6 in case of mice) samples. From (55).

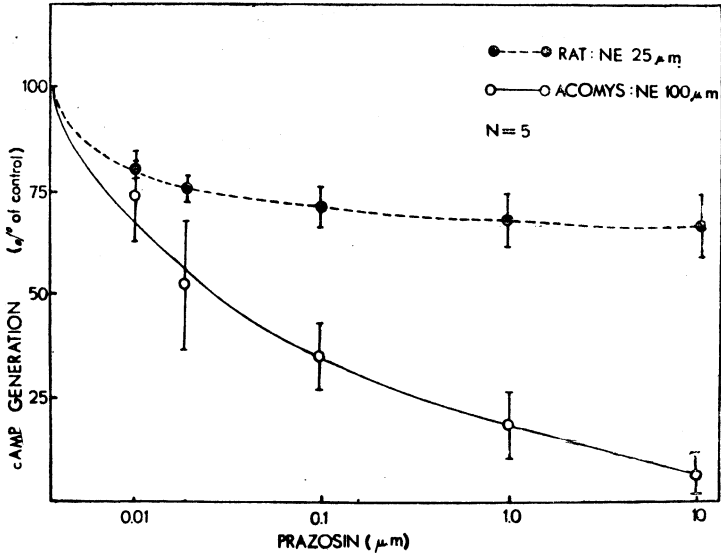


Fig. 8. High efficiency of inhibitory action of prazosin on noradrenaline (NE)-stimulated cyclic AMP generation in the cerebral cortical slices of the *Acomys cahirinus* in comparison with the rat. The effect of 100  $\mu\text{M}$  (for *Acomys*) or 25  $\mu\text{M}$  (for rat) noradrenaline (% of conversion in stimulated sample — basal % of conversion) was assumed to be 100%. Each bar represents the mean  $\pm$  SEM of 5-6 experiments carried out in duplicates. Even the effect of low concentration of noradrenaline is inhibited by prazosin by no more than 25% in the rat, while in the *Acomys* the blockade is complete. (From 16).

es in this homeostasis. An increase in responsiveness to agonists would counteract to some extent the decline in calcium availability, as increased formation of inositol polyphosphates may facilitate both the mobilisation of the intracellular calcium stores and the external calcium influx.

#### *Comparison of receptor systems in various animal species*

Most receptor studies, whether carried out by radioligand binding or by second messenger assay, are carried out on rats. They are often generalized to other species, particularly to man, but comparative studies indicate that the responses of second messenger systems may vary from species to species. Thus, the response of the cyclic AMP generating system to noradrenaline in the rat is much stronger than in the guinea pig; in the latter species the response to histamine is much greater than that to noradrenaline (40).

We carried out comparative studies using rats and a novel laboratory species, the spiny mouse *Acomys cahirinus* (55).

The responsiveness of  $\alpha_1$ -adrenoceptor was compared in the spiny mouse, rat and mouse by IP assay. The maximum response of the phosphatidylinositol system in the *Acomys* was significantly higher than in other species, although the affinity of the receptors seemed to be similar in all three species (Fig. 7). This notion was confirmed by demonstration that prazosin similarly inhibits the response to noradrenaline in the *Acomys* and in the rat, which suggested greater efficiency of the  $\alpha_1$ -adrenergic system in the *Acomys*. In fact, the behavioral observations agree with this conclusion.

Studies with the  $\beta$ -adrenoceptor system, employing cyclic AMP assay, showed important differences between the rat and the spiny mouse. Although the basal cyclic AMP levels in both species were the same, the responsiveness to noradrenaline was more than twice as high in the rat. Even more dramatic was the difference in the effect of isoproterenol: the classic  $\beta$ -adrenoceptor agonist did not produce a significant accumulation of cyclic AMP over the basal level in cortical slices of *Acomys*. In other experiments only a very weak effect was observed in this species. It should be noted that other studies did not reveal a lower density

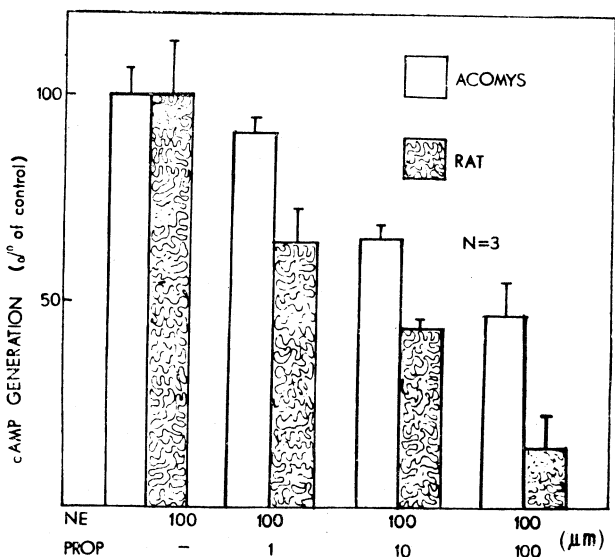


Fig. 9. Higher efficiency of inhibitory action of propranolol (PROP) on noradrenaline (NE)-stimulated cyclic AMP generation in the cerebral cortical slices of the rat in comparison with *Acomys*. The net stimulatory effect of 100  $\mu$ M noradrenaline (% of conversion in stimulated sample — basal % of conversion) was assumed to be 100%. Each bar represents the mean  $\pm$  SEM of 5 — 6 experiments carried out in duplicates. Even equimolar concentrations of propranolol do not inhibit cyclic AMP generation in slices from *Acomys* by more than 50% (From 16).

of  $\beta$ -adrenoceptor in the cortex of *Acomys*, but on the contrary, their density was higher by ca. 20% (14). This result shows that, at least for trans-species comparison, the density of receptors cannot be regarded as an index in the respective reactivity of the system.

Further characterization of adrenoceptor-linked cyclic AMP system in the spiny mouse revealed that in fact the system seems to be more dependent on  $\alpha_1$ -adrenoceptors than in the rat (14). Thus,  $\alpha_1$ -adrenergic antagonist, prazosin, which in maximum concentrations inhibits only about 25% of noradrenaline stimulatory effect in the rat, in the spiny mouse may completely abolish the cyclic AMP system response to noradrenaline (Fig. 8). On the other hand, while in the rat the inhibition of  $\beta$ -adrenoceptor by propranolol leads to almost complete inhibition of the cyclic AMP response to noradrenaline, in the spiny mouse the inhibitory effect is only partial (Fig. 9).

Thus, studies on receptor responses by measuring the formation of second messengers seem to reveal several features which cannot be discovered by receptor binding techniques. In our opinion they are presently the most suitable biochemical methods for the assessment of reactivity of the systems of metabotropic receptors.

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