Biochemical Pharmacology, Vol. 39, No. 3, pp. 439–444, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

LITHIUM BUT NOT CHOLINERGIC LIGANDS INFLUENCE GUANYLATE CYCLASE ACTIVITY IN INTACT HUMAN LYMPHOCYTES

T. SCHUBERT and W. E. MÜLLER*

Department of Psychopharmacology, Central Institute of Mental Health, D-6800 Mannheim, Federal Republic of Germany

(*Received* 31 *January* 1989; *accepted* 23 *August* 1989)

Abstract—The presence of guanylate cyclase in intact circulating human lymphocytes and the sensitivity of this enzyme to stimulation by sodium nitroprusside could be confirmed. However, in contrast to other observations all attempts failed to stimulate the enzyme by cholinergic agonists, despite the use of M1 as well as M2 selective agonists. These findings do not support the assumption that cholinergic recognition sites on human lymphocytes described by many groups are part of a functioning muscarinic transducing mechanism. While several other neuroreceptor agonists were also unable to affect lymphocyte guanylate cyclase activity, lithium was found to potently inhibit the stimulation of guanylate cyclase by sodium nitroprusside at an intracellular concentration close to the therapeutic plasma levels. It is suggested that the effects of lithium on guanylate cyclase activity in human lymphocytes could be related to a possible mechanism of action of lithium in affective disorders.

Because of the inaccessibility of the central cholinergic neurotransmission to experimental investigations in man, we are currently investigating the possible use of intact circulating human lymphocytes as cellular model systems to study cholinergic functions in man [1, 2]. Peripheral human lymphocytes possess a number of characteristics of cholinergic neurons, e.g. membrane bound acetylcholinesterase [3] and muscarinic cholinergic recognition sites [1, 4–6]. Moreover, several reports suggest that cholinergic recognition sites on human lymphocytes are part of a cholinergic transducing mechanism, since cholinergic agonists like acetylcholine lead to an intracellular accumulation of c-GMP [7, 8] and augment lymphocyte mediated cytotoxicity, similar to the effects of lectins [9]. Since lectins also augment intracellular c-GMP concentration [10] it has been suggested that both effects on cytotoxicity are mediated by an increase of intracellular c-GMP. These findings suggest the important role of guanylate cyclase for cholinergic signal transduction in intact circulating human lymphocytes. Accordingly, we investigated the general properties of this enzyme in human lymphocytes and its possible stimulation by cholinergic agonists. Moreover, we investigated the possible effects of lithium on guanylate cyclase activity in this system.

MATERIALS AND METHODS

Materials. 8-[^{14}C]Guanosine 3′,5′-cyclic phosphate (sp. act. 52 mCi/mmol) was from Amersham-Buchler (Braunschweig, F.R.G.), guanine-8-[^{3}H]hydrochloride (sp. act. 10.5 Ci/mmol) from Rotem Industries (Beer-Sheva, Israel). Dulbecco's modified Eagle's medium (DME, Hepes buffered) was obtained from Sigma-Chemie (Deisenhofen, F.R.G). All other chemicals used were of analytical grade. Drugs were obtained from the manufacturers. Incubation media were always freshly prepared and filtered through sterile Millex-GS 0.22 μm filter units (Millipore S.A.; Molsheim, France) before use. Cells were counted on a Coulter Counter T 540.

Lymphocyte preparation. Human lymphocytes were isolated from fresh blood (EDTA containers) of healthy adult donors (aged between 23 and 35 years) using Ficoll-Paque (Pharmacia, Uppsala, Sweden) according to the method of Böyum [11] and the directions provided by the manufacturer. The washed lymphocytes were finally suspended in DME medium containing 5% autologous serum at a concentration of 6×10^6 cells/mL. Viability as determined by the trypan blue exclusion test was 95% or more. Thrombocytes concentration was less than $10^4/\mu$L. The pH of the incubation medium was adjusted to 7.35 and did not exceed 7.5 during incubations.

Guanylate cyclase assay. Guanosine 3′,5′-cyclic phosphate (c-GMP) was assayed slightly modified, as previously described [7, 12]. Briefly, the lymphocyte suspension was incubated at 37° for 2 hr with [^{3}H]guanine in a concentration of 1 μCi/4×10^6 cells. After this incubation time [^{3}H]guanine incorporation into GTP was maximal. Aliquots (0.5 mL) of the labelled cell suspension, containing approximately 3×10^6 cells were added to plastic tubes and again incubated for 10 min. Theophylline was added 20 min and lithium chloride 10 min before the addition of the various drugs. The reactions were terminated by the addition of ice-cold TCA (final concentration 10%) and sonication for 30 sec (Sonorex Rk 100, Bandelin). After the addition of 8-[^{14}C]c-GMP as internal standard, the samples were passed through a column (1.0 × 10 cm) of Dowex 50W X2–

* Correspondence to: Prof. Dr W. E. Müller, Abteilung Psychopharmakologie Zentralinstitut für Seelische Gesundheit, D-6800 Mannheim, F.R.G.

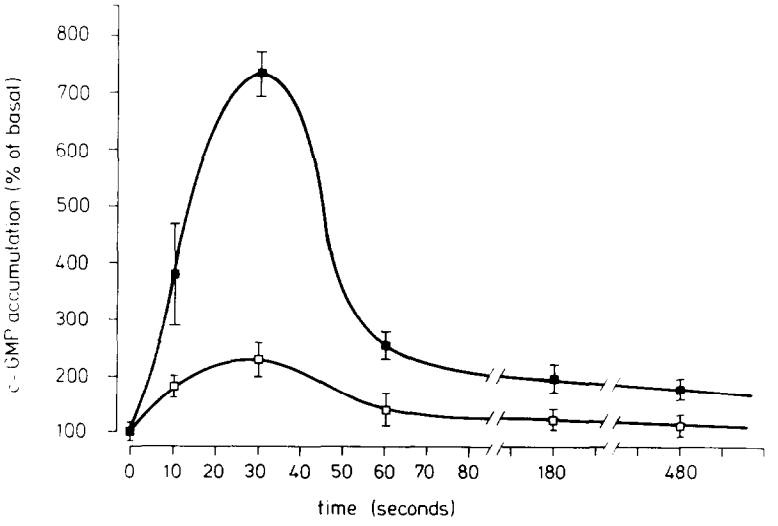

Fig. 1. Time course of the sodium nitroprusside induced intracellular c-GMP accumulation. Lymphocytes were incubated in the absence of theophylline with sodium nitroprusside at concentrations of 1 mmol/L (■) or 0.1 mmol/L (□) for the time indicated. The samples were processed as described in Materials and Methods. Values represent means ± SD of three experiments.

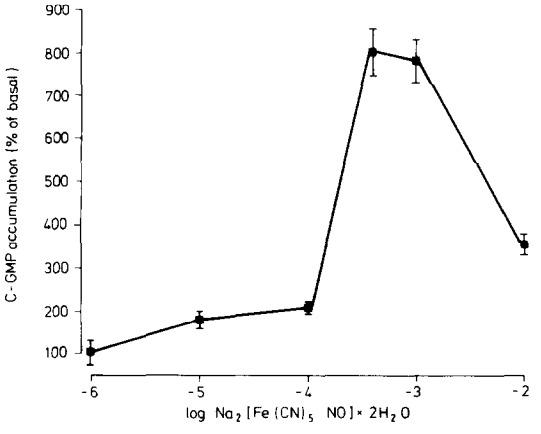

Fig. 2. Concentration dependence of the sodium nitroprusside induced intracellular c-GMP accumulation. Lymphocytes were incubated with sodium nitroprusside in the concentrations indicated. The incubation was terminated after 30 sec and c-GMP was determined as described in Materials and Methods. Values represent means ± SD of three experiments.

400, applied to a Baker SPE system (J. T. Baker, Groß-Gerau, F.R.G.). First, labelled GTP and GDP were together eluted with 5 mL 0.1 N HCl and 2.5 mL of water, before labelled c-GMP was eluted with 1.2 mL of water. To separate c-GMP from small amounts of GDP (less than 1% of total GDP was found in the c-GMP fraction as evaluated by HPLC) we used a precipitation step. Twenty μL of 3.2 mol/L $ZnSO_4$ and 20 μL of 3.2 mol/L Na_2CO_3 solutions were added to each tube. The supernatant was removed and assayed for ^3H and ^{14}C. Eight mL of Quickszint 2000 (Zinsser, F.R.G.) were added and the samples were counted by a double-label program on a Beckman LS 6800 liquid scintillation counter. The recovery of c-GMP was always about 70%.

Data were analysed either as radioactivity found in c-GMP expressed as percentage of the basal value or by calculating the ratio of [^{3}H]c-GMP to the radioactivity found in GTP, GDP and c-GMP. The latter method avoids differences in [^{3}H]guanine incorporation in the GTP/GDP system between individual blood donors and in repeated experiments with blood from the same donor, in which case the calculated ratios did not change significantly over time.

Lithium uptake in lymphocytes. Lymphocytes suspended in assay medium at concentration of 2×10^7 cells/mL, were preincubated for 1 hr at 37°. To 0.5 mL of the lymphocyte suspension, lithium chloride was added in two different concentrations and the incubation was continued for the times indicated. The lymphocyte suspensions were put on ice for 30 sec and centrifuged for 3 min at 1500 *g* by 4°. The incubation medium was quickly removed and the lymphocytes were washed three times by suspending in ice-cold Hank's balanced salt solution and centrifuged (see above). To assure that no lymphocytes were lost during the washing procedure the lymphocyte concentration of the washing solutions were determined with a Coulter Counter T 540. Because small amounts of lithium ions are bound extracellularly at the cell membrane and at the plastic tubes used, the values were corrected by subtracting a blank value. Blank values were generated by the addition of lithium chloride to the lymphocyte suspension at 4° and the immediate start of the washing procedure. Finally, the lymphocytes were lysed by the addition of TCA in a concentration of 10% and sonication for 10 min. The samples were contrifuged at 4000 *g* for 10 min and lithium was quantified by flame photometry. For calculating intracellular lithium concentrations an average lymphocyte volume of 100 fL/lymphocyte was assumed.

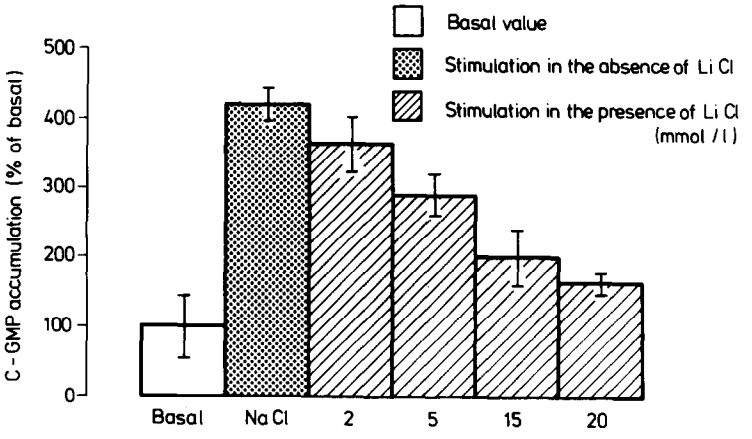

Fig. 3. Inhibitory effect of lithium chloride on the sodium nitroprusside induced intracellular c-GMP accumulation. Lymphocytes were preincubated for 10 min with lithium chloride in the indicated concentrations. After preincubation, sodium nitroprusside was added (final concentration 1 mmol/L) and the incubation was stopped after 30 sec. Lithium chloride alone or sodium chloride (as control) did not alter basal guanylate cyclase activity. c-GMP was determined as described in Materials and Methods. Values represent means ± SD of three experiments.

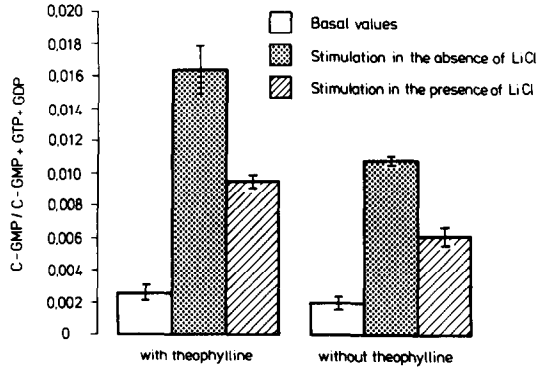

Fig. 4. Influence of theophylline on sodium nitroprusside induced intracellular c-GMP accumulation in the presence or absence of lithium chloride. Lymphocytes were preincubated for 20 min with or without theophylline (10 mmol/L) and for 10 min with lithium chloride or sodium chloride respectively (control), both at a concentration of 20 mmol/L. After the addition of sodium nitroprusside (1 mmol/L) the incubation was continued for 30 sec. c-GMP and GTP + GDP were determined as described in Material and Methods. The data represent means ± SD of three experiments.

RESULTS

General properties of guanylate cyclase activity in intact human lymphocytes

After incubation with [^{3}H]guanine but without the addition of a stimulator of guanylate cyclase only very low levels of [^{3}H]c-GMP were found in human lymphocytes. However, in agreement with the report by Atkinson *et al.* [13] we were able to augment c-GMP levels by nearly one order of magnitude by stimulation of guanylate cyclase activity [14] with sodium nitroprusside (Fig. 1). The response of lymphocyte guanylate cyclase to low and high concentrations of sodium nitroprusside was very rapid with an apparent maximum already observed after only 30 sec (Fig. 1). The activation of lymphocyte guanylate cyclase activity by sodium nitroprusside was dependent on the medium concentration of the drug probably in a biphasic fashion with a slight increase up to sodium nitroprusside concentrations of about 100 μmol/L and a steep increase between 0.1 and 1.0 mmol/L (Fig. 2). Half-maximal stimulation was observed at about 500 μmol/L (Fig. 2).

Effects of cholinergic agonists and other transmitter substances

In contrast to the pronounced stimulation seen for sodium nitroprusside (Figs. 1 and 2) we were unable to elevate baseline levels of c-GMP in intact human lymphocytes by a variety of cholinergic agonists using many different concentrations and different stimulation times (Table 1). Similar negative results were also obtained after inhibition of phosphodiesterase by preincubating the lymphocytes with theophylline. Several other neurotransmitters or receptor agonists including norepinephrine, serotinin, apomorphine, histamine and neurotensine, which have been shown to stimulate guanylate cyclase activity in other tissues [4, 15–19] or which possess specific binding sites on human lymphocytes did not elevate c-GMP levels in human lymphocytes for the conditions indicated (Table 1). Moreover, preincubation of human lymphocytes with norepinephrine, serotonin, histamine as well as carbachol did not modify the stimulation of lymphocyte guanylate cyclase activity by sodium nitroprusside (Table 1).

Effects of lithium

Incubating human lymphocytes with lithium chloride up to concentrations of 20 mmol/L did not alter baseline levels of c-GMP (data not shown). However the activation of guanylate cyclase activity by sodium nitroprusside was markedly inhibited by lithium chloride in a dose dependent fashion (Fig.

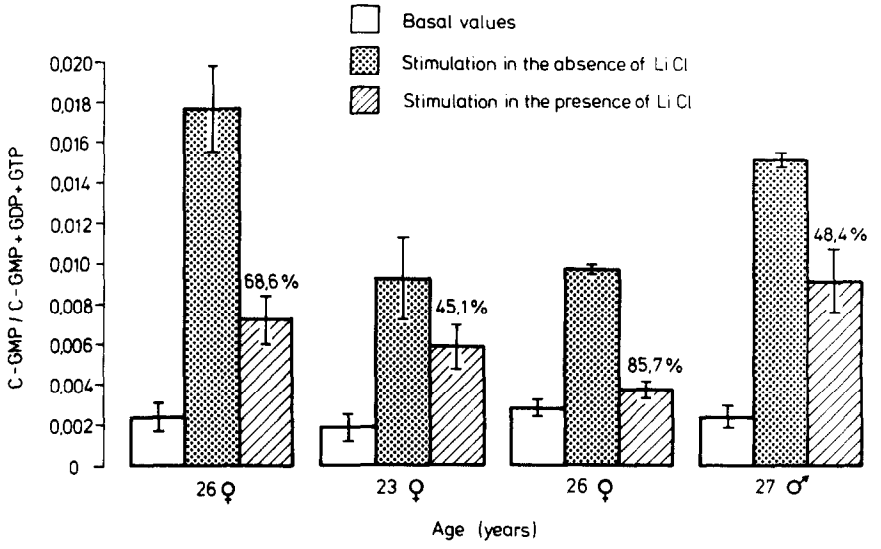

Fig. 5. Individual variations in the activation of guanylate cyclase by sodium nitroprusside in the presence and absence of lithium chloride. Lymphocytes of four healthy subjects were incubated with sodium nitroprusside (concentration 1 mmol/L) for 30 sec in the presence of lithium chloride or sodium chloride (concentration of 20 mmol/L) respectively (control). Lithium chloride and sodium chloride were added 10 min before the addition of sodium nitroprusside. c-GMP and GTP + GDP were determined as described in Materials and Methods. Data represent means ± SD of three experiments.

Table 1. The insensitivity of lymphocyte guanylate cyclase to cholinergic agonists and other neuroreceptor agonists. Summary of the experimental conditions investigated, which failed to give any effect on baseline levels of c-GMP or on c-GMP levels after stimulation with sodium nitroprusside

Substance	Concentration investigated	Time of stimulation (min)
Effects on basline c-GMP		
Carbachol	5 mmol/L	2
	1 mmol/L	0.15, 0.5, 1, 2, 3, 5, 10, 20, 50
	10 μmol/L	2
	1 nmol/L	2
Oxotremorine	10 μmol/L	0.5, 3
	1 μmol/L	0.5, 2, 3, 5
	100 nmol/L	0.5, 2
	10 nmol/L	0.5, 2
McN-A-343	1 mmol/L	0.15, 2
	100 μmol/L	0.5, 1, 2, 3
	100 pmol/L	2
Neurotensine	25 μmol/L	0.5, 2
	1 μmol/L	0.5
Norepinephrine	100 μmol/L	5, 10
Serotonin	10 μmol/L	0.5, 2
Histamine	100 μmol/L	0.5, 2
Apomorphine	100 μmol/L	0.5, 2
Effects on stimulation by sodium nitroprusside		
Carbachol	1 mmol/L	0.5, 2
Norepinephrine	100 μmol/L	0.5, 2
Serotonin	100 μmol/L	0.5, 2
Histamine	100 μmol/L	0.5, 2

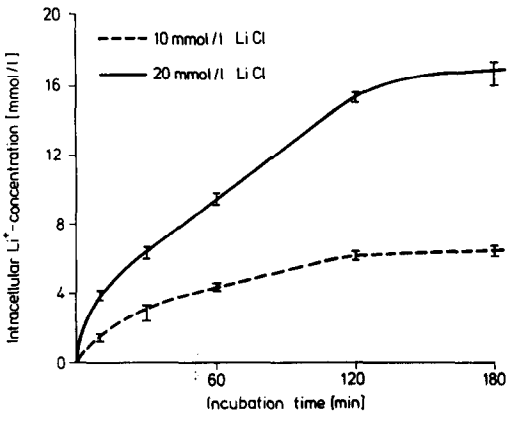

Fig. 6. Time course of lithium-uptake by lymphocytes. Lymphocytes were incubated for the indicated time with lithium chloride at concentrations of 10 or 20 mmol/L. About 1–2 × 10^7 lymphocytes were used per sample. The intracellular lithium concentration was evaluated as described in Materials and Methods. Data represent means ± SD of two experiments.

3). Half-maximal inhibition was observed at about 10 mmol/L lithium chloride (Fig. 3). To further elucidate the inhibitory effect of lithium, we also performed several experiments after preincubation of lymphocytes with theophylline (Fig. 4). As expected, inhibition of phosphodiesterase by theophylline increased the levels of c-GMP in intact human lymphocytes after stimulation with sodium nitroprusside (Fig. 4). However, the inhibitory effect of lithium was not different whether the lymphocytes were preincubated with theophylline or not (about 50% inhibition, Fig. 4). The activation of lymphocyte guanylate cyclase activity by sodium nitroprusside and inhibition of this effect by lithium showed a considerable variation when investigated in individual healthy subjects (Fig. 5). There was no correlation between both effects (Fig. 5). However, when the same experiment was performed with lymphocytes of the same subject, the individual responses to sodium nitroprusside and/or lithium chloride were always comparable (data not shown).

Estimation of the intracellular lithium concentration

In order to have an estimation about the intracellular lithium concentrations under the experimental conditions employed, we investigated the time course of lithium uptake by intact human lymphocytes for two lithium chloride concentrations (Fig. 6). Intracellular lithium concentrations increased over the time and tended to reach equilibrium after 3 hr of incubation (Fig. 6). Based on these data, the intracellular lithium concentration after 10 min incubation with lithium chloride concentrations of 10 or 20 mmol/L (the standard conditions used for the experiments) were estimated as about 1.5 or 4 mmol/L, respectively.

DISCUSSION

The data presented in the present communication confirm previous findings about the presence of guanylate cyclase activity in intact circulating human lymphocytes [13]. In contrast to some other authors [4, 8] but in agreement with the preliminary observations by Atkinson *et al.* [13], all our attempts to stimulate lymphocyte guanylate cyclase by cholinergic agonists failed in spite of the use of rather M1 selective compounds like McN-A-343 as well as rather M2 selective compounds like oxotremorine or carbachol. At the present state of the experiments, we are not able to explain these discrepant findings, especially since our experiments with sodium nitroprusside clearly show that functioning guanylate cyclase is present. Although today most findings point against the concept of c-GMP being a critical second messenger of *m*-cholinoceptors, strong evidence is present that in many tissues activation of *m*-cholinoceptors leads to an activation of guanylate cyclase as an event secondary to other mechanisms, possibly an increase of intracellular calcium [20]. Accordingly, the original observations about the stimulation of lymphocyte guanylate cyclase activity by cholinergic agonists strongly suggested the presence of cholinergic functions in intact human lymphocytes [4, 8]. Our failure to find any effect of cholinergic agonists on lymphocyte guanylate cyclase activity does not support the assumption that cholinergic recognition sites found on human lymphocytes by many groups [1, 4–6] are part of a functioning muscarinic transducing mechanism. Thus, the suitability of intact human lymphocytes to study cholinergic mechanisms in man is not supported by our findings. Our failure to find cholinergic functions on human lymphocytes in the presence of binding sites with properties fairly similar but not identical to those of cholinergic recognition sites [1, 4–6] confirms the critique on the use of binding studies as sole evidence for receptors [21, 22].

Considering the insensitivity of lymphocyte guanylate cyclase to cholinergic agonists and many other neuroreceptor related molecules, the pronounced effect of lithium on the stimulation of the enzyme by sodium nitroprusside is remarkable. While our observations with theophylline strongly indicate that the decreased levels of c-GMP are not related to an activation of phosphodiesterase, the possible mechanism by which lithium inhibits guanylate cyclase activity remains obscure. In contrast to the well known effects of lithium on hormone-stimulate adenylate cyclase activity in most tissues investigated [23] very little is known about possible effects of lithium on guanylate cyclase activity. Zatz [24] reported that lithium is able to inhibit the increase of c-GMP in rat pineal glands after stimulation by norepinephrine. Similarly, Belmaker *et al.* [25] reported that lithium-treatment prevented the rise in plasma c-GMP after the subcutaneous application of epinephrine in manic-depressive patients. Although the pharmacological significance of these observations was not further investigated, both agree with our findings suggesting a direct inhibition of guanylate cyclase by lithium in spite of the mechanism of stimulation.

If an inhibition of guanylate cyclase by lithium contributes to its therapeutic efficacy in effective disorders is not known although the effect takes

place at intracellular concentrations quite close to the therapeutic plasma concentrations (around 1.5 mmol/L) (this paper) or at similar plasma levels [25] in the case of the epinephrine-stimulation of plasma c-GMP. Moreover, carbamazepine, which like lithium is used to treat bipolar patients, reduces the c-GMP levels in the liquor [26] and also inhibits the sodium nitroprusside induced increase of c-GMP in human lymphocytes (Schubert and Müller, unpublished observations). Thus, the similar effect of both drugs on guanylate cyclase activity represents an attractive clue for a possible common mechanism of action of both drugs in affective disorders. Moreover, the pronounced individual response of human lymphocytes for the inhibitory effect of lithium on guanylate cyclase suggests the possibility of using this system to test the individual responsiveness of patients to the pharmacological effects of lithium.

REFERENCES

1. Bering B, Moises HW and Müller WE, Muscarinic cholinergic receptors on intact human lymphocytes. Properties and subclass characterization. *Biol Psychiat* **22**: 1451–1458, 1987.
2. Moises HW, Bering B and Müller WE, Personality factors predisposing to depression correlate significantly negatively with M_1-muscarninic and β-adrenergic receptor densities on blood cells. *Eur Arch Psychiatr Neurol Sci* **237**: 209–217, 1988.
3. Szelényi JG, Bartha E and Holan SR, Acetylcholinesterase activity of lymphocytes: an enzyme characteristic of T-cells. *Br J Haematol* **50**: 241–245, 1982.
4. Zalcman SJ, Neckers LM, Kaayalp O and Wyatt RJ, Muscarinic cholinergic binding sites on intact human lymphocytes. *Life Sci* **29**: 69–73, 1981.
5. Adam A, Nordberg A and Slanina P, A muscarinic receptor type in human lymphocytes: a comparison of ^{3}H-QNB binding to intact lymphocytes and lysed lymphocyte membranes. *Life Sci* **38**: 1359–1368, 1986.
6. Rabey JM, Shenkman L and Gilad GM, Cholinergic muscarinic binding by human lymphocytes: changes with aging, antagonist treatment, and senile dementia of the Alzheimer type. *Ann Neurol* **20**: 628–631, 1986.
7. Haddock AM, Patel KR, Alston WC and Kerr JW, Response of lymphocyte guanyl cyclase to propanolol, noradrenaline, thymoxamine and acetylcholine in extrinsic bronchial asthma. *Br Med J* **2**: 357–359, 1975.
8. Iliano G, Tell GPF, Siegel MI and Cuatrecasas P, Guanosine 3′:5′-cyclic monophosphate and the action of insuline and acetylcholine. *Proc Natl Acad Sci USA* **70**: 2443–2447, 1973.
9. Strom TB and Carpenter CB, Cyclic nucleotides in immunosuppression—neuroendocrine pharmacologic manipulations and *in vivo* immunoregulation of immunity acting via second messenger systems. *Transplant Proc* **12**: 304–310, 1980.
10. Hadden JW, Hadden EM, Haddox MK and Goldberg ND, Guanosine 3′,5′-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. *Proc Natl Acad Sci USA* **69**: 3024–3027, 1972.
11. Böyum A, Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Invest* **21**: 77–89, 1968.
12. Richelson E, Prendergast FG and Divinetz-Romero S, Muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells of: ionic dependence and effects of local anesthetics. *Biochem Pharmacol* **27**: 2039–2048, 1978.
13. Atkinson JP, Kelly JP, Weiss A, Wedner HJ and Parker CW, Enhanced intracellular c-GMP concentrations and lectin-induced lymphocyte transformation. *J Immunol* **121**: 2282–2291, 1978.
14. Katsuki S, Arnold W, Mittal C and Murad F, Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cycl Nucl Res* **5**: 31–41, 1979.
15. Schwartz JP, Catecholamine-mediated elevation of cyclic-GMP in the rat C-b glioma cell line. *J Cycl Nucl Res* **2**: 287–296, 1976.
16. Vethoshkin AV, Fomenko AM and Zozulia AA, Lymphocyte serotonin receptors: a radioreceptor study (translation). Biull Eksp Biol Med **94**: 52–53, 1982.
17. LeFur G, Phan T and Uzan A, Identification of stereospecific ^{3}H-spiperone binding sites in mammalian lymphocytes. *Life Sci* **26**: 1139–1148, 1980.
18. Richelson E, Histamine H_1 receptor-mediated guanosine 3′,5′-monophosphate formation by cultured mouse neuroblastoma cells. *Science* **201**: 69–71, 1978.
19. Amar S, Mazella J, Checler F, Kitagbi P and Vincent JP, Regulation of cyclic GMP levels by neurotensin in neuroblastoma clone N1E115. *Biochem Biophys Res Commun* **129**: 117–125, 1985.
20. Dunwiddie TV and Hoffer BJ, The role of cyclic nucleotides in the nervous system. In: *Cyclic Nucleotides* 2 (Eds. Kebabian JW and Nathanson JA), pp. 389–445. Springer, Berlin, 1982.
21. Maloteaux JM, Waterkein C and Laduron PM, Absence of dopamine and muscarinic receptors on human lymphocytes. *Arch Int Pharmacodyn* **258**: 174–176, 1982.
22. Gossuin A, Maloteaux JM, Trouet A and Laduron PM, Differentiation between ligand trapping into intact cells and binding on muscarinic receptors. *Biochim Biophys Acta* **804**: 100–106, 1984.
23. Bunney WE and Garland-Bunney BL, Mechanisms of action of lithium in affective illness: basic and clinical implications. In: *Psychopharmacology: The Third Generation of Progress* (Ed. Meltzer HY), pp. 553–565. Raven Press, New York, 1987.
24. Zatz M, Low concentrations of lithium inhibit the synthesis of cyclic AMP and cyclic GMP in the rat pineal gland. *J Neurochem* **32**: 1315–1321, 1979.
25. Belmaker RH, Kon M, Ebstein RP and Dasberg H, Partial inhibition by lithium of the epinephrine-stimulated rise in plasma cyclic GMP in humans. *Biol Psychiat* **15**: 3–8, 1980.
26. Post RM, Ballenger JC, Uhde TW, Smith C, Rubinow DR and Bunney WE, Effect of carbamazepine on cyclic nucleotides in CSF of patients with affective illness. *Biol Psychiat* **17**: 1037–1045, 1982.