Category of lesion	n	Right CA	Left CA	Level of significance
I Unilateral PL only	27	1.33 ± 0.91	0.31 ± 0.29	$p < 0.005$
II Unilateral PL part of PI	13	1.27 ± 0.76	0.50 ± 0.35	$p < 0.01$
III Unilateral or bilateral PI	12	0.34 ± 0.27	0.41 ± 0.31	NS
IV No apparent abnormality	29	0.53 ± 0.56	0.50 ± 0.54	NS
V Unoperated controls	20	0.66 ± 0.51	0.68 ± 0.47	NS

The effect of selective lesions in the brain on corpus allatum activity. Gland activity is expressed in pmol juvenile hormone synthesized per hour (means ± standard deviation). The right gland was always on the operated side, while the left gland was used as control. The activities of the two glands in each individual were statistically analyzed using the Wilcoxon matched pairs signed ranks test. CA, corpus allatum; NS, not significant; PI, pars intercerebralis; PL, pars lateralis.

II the pars lateralis plus part of the pars intercerebralis were impaired
III more than half or the total number of M-NSC were destroyed but the L-NSC appeared intact
IV no abnormality was apparent, or the damage was restricted to the perineurium.

The results are displayed in the table where values from unoperated controls are also included. In categories I and II a highly significant increase in the mean rate of juvenile hormone biosynthesis by one of the glands of a pair was observed. In categories III and IV, and in the unoperated controls, the mean activities of both glands of a pair remained similar and relatively low. In previous studies, we observed that corpora allata from beetles reared under short-day conditions displayed relatively low rates of juvenile hormone biosynthesis at emergence, and were gradually inactivated just before diapause[13]. However, when the corpora allata of short-day beetles at emergence were denervated, and the animals transferred to long-day and simultaneously starved for 3 days, the glands became activated[4]. Intact glands remained restrained in their activity under these conditions. In the present paper, a similar experimental protocol has been followed to investigate whether selective cauterization could lead to gland activation. The results in the table show that with lesions whereby the pars lateralis area was unilaterally damaged (categories I and II), the gland on the ipsilateral side became activated while the contralateral corpus allatum remained restrained in its activity. The implication of this finding is that a corpus allatum inhibitory center is located in the pars lateralis, since gland inhibition is removed by selective cauterization of this region. Taking the innervation of the corpus allatum into consideration, this evidence provides strong support for our previous suggestions that the L-NSC produce an allatostatin in the Colorado beetle[7].

Surprisingly, removal of the M-NSC did not appear to affect corpus allatum activity. However, we cannot exclude the possibility that under different experimental conditions these cells may indirectly influence gland activity. In *D. punctata*, cauterization of the L or M-NSC led to gland activation[9]. In this cockroach, it has been postulated that the L-NSC do not inhibit the gland directly but provide input into the contralateral M-NSC which produce and release an allatostatin through the neural pathway. Whether this essential difference between the beetle and the cockroach is related to the different pattern of corpus allatum innervation and activity in the two species remains to be seen. Another intriguing aspect is that the precise role of the L-NSC, whether inhibitory or stimulatory, also appears to be species dependent. For example, in the locusts *Schistocerca paranensis*[15] and *Locusta migratoria*[16], the evidence implies that an allatotropin is produced by the L-NSC. A satisfactory explanation for such diverse observations in different species must await further experimentation, including isolation and identification of the substance(s) produced by the L-NSC.

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Hormone and forskolin-stimulated cyclic AMP accumulation in human lymphocytes: reliability of longitudinal time measurements[1]

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Summary. Reliability of measurement of lymphocyte cyclic AMP synthesis in intact cells was estimated by taking 3 successive blood samples during a one-month period from 11 healthy volunteers. Isoproterenol and prostaglandin E_1-stimulated cyclic AMP accumulation were used to evaluate the activity of these two receptor activities in human lymphocytes. Forskolin-stimulated cyclic AMP accumulation was used to evaluate the activity of the Ns/catalytic subunit. Only for forskolin was significant reliability observed. For isoproterenol and prostaglandin E_1 significant reliability was observed only for male subjects.
Key words. Cyclic AMP synthesis; measurement in lymphocytes; forskolin; isoproterenol; prostaglandin E_1.

The easy accessibility of lymphocytes, and the non-invasive procedures required for their isolation from human subjects, make this tissue an attractive model for studying hormone-stimulated adenylate cyclase activity in man[2-11,20,21]. Widespread use of this tissue in human investigations suggests that a study of the reliability of successive, longitudinal measurements of cyclic AMP accumulation in individual subjects is worthwhile. After the reliability of repeated measurements has been established in a general population sample, it will be possible to examine more meaningfully variations of lymphocyte adenylate cyclase activity as a result of disease or pharmacological intervention.

The hormone-stimulated adenylate cyclase complex consists of at least three principal components[12]. The receptor spans the plasma membrane and its external side provides a site for binding circulating hormones. The Ns and Ni proteins, or guanine-nucleotide binding regulatory subunits, link the receptor to the catalytic subunit of the enzyme. The activated or inhibited (in the case of Ni coupling) catalytic subunit converts ATP to cyclic AMP. The various components of the hormone-sensitive adenylate cyclase complex can to some extent be separately measured and a number of studies have demonstrated the functional independence of these subunits[13-15].

Three blood samples were obtained from 11 healthy volunteers over a 30-day period. Isoproterenol, a beta-adrenergic agonist, and prostaglandin E_1-stimulated cyclic AMP accumulation were used to evaluate the activity of these two receptor activities in human lymphocytes. Forskolin-stimulated[16] cyclic AMP accumulation was used to evaluate the activity of the Ns/catalytic subunit.

Materials and methods. All of the subjects (7 women and 4 men), average age 31 years (19–38), were healthy and taking no medication for at least two weeks prior to the study. Blood samples were taken between 08.00 and 09.30 h while the subject was upright, in a sitting position. Three blood samples were obtained from each subject over the course of a 1-month period. The subjects consisted of hospital personnel, free of medical pathology and with no history of heart disease, hypertension, diabetes or asthma. Blood pressure was measured with a standard cuff manometer using Korotkoff phase 5 sound in measuring diastolic blood pressure: both diastolic and systolic pressure were in the normal range for all subjects. No attempt was made to restrict diet or salt intake for these individuals.

Lymphocytes were isolated from 50 ml of heparinized blood using the method of Boyum[17], modified in this laboratory as follows; to reduce platelet contamination to minimal levels, after the initial ficoll purification step lymphocytes are repeatedly centrifuged at low speed (250 × g) until microscopic examination shows greater than 95% lymphocyte purity. An additional centrifugation over ficoll is often necessary to reduce platelet and red blood cell contamination. One ml aliquots of the purified lymphocyte suspension were preincubated for 30 min at 37 °C in a standard balanced salt solution (pH 7.6) containing the following components: 0.13 M NaCl, 0.01% glucose, 1% bovine serum albumin, 0.005 mM $CaCl_2$, 0.098 mM $MgCl_2$, 0.54 mM KCl, 0.015 M Tris, titrated with HCl to pH 7.6. The lymphocytes were then incubated for an additional 20 min in the presence and absence of drug. 3-isobutyl-1-methylxanthine (1 mM), a potent phosphodiesterase inhibitor[18], was present throughout. Cyclic AMP accumulation was linear under these conditions for at least 20 min. Forskolin (60 μM), isoproterenol (10 μM) and prostaglandin E_1 (1 μM) significantly increase cyclic AMP accumulation above basal activity in this assay procedure. Lymphocytes were collected by centrifugation, suspended in 1 ml ethanol and disrupted with a polytron homogenizer. Cyclic AMP was determined in duplicate by protein binding assay[19]. All drugs were used at concentrations previously shown to be maximally stimulating under the conditions employed in this assay[20,21].

Reliability of successive measurements was estimated by one-way ANOVA (repeated measures): cyclic AMP accumulation within the three samples from each subject was compared to this variable between subjects. All the data manipulations were carried out using NWA-STATPAK (Portland, Oregon) and Lotus Symphony on an IBM PC. Abbreviations employed: F = F ratio, the mean square value (msv) of the between group variance divided by the msv of the within group variance; DF = degrees of freedom.

Results and discussion. Basal cyclic AMP accumulation in lymphocytes obtained from three repeated blood samples, from each of the 11 subjects in the study, is shown in figure 1. Reliability of successive measurements was estimated by one-way ANOVA (repeated measures). A significant difference was observed for cyclic AMP accumulation within the three samples from each subject, compared to this variable between subjects (F = 2.36; DF = 10,20; p = 0.049; between subjects mean square value or msv = 8.82 and within subjects msv = 3.75). The significance levels for male and female subjects were: men (F = 6.15; DF = 3,6; p = 0.082; msv between = 12.88 and msv within = 2.09), women (F = 1.59; DF = 6,12; p = 0.23.; msv between = 8.14 and msv within = 5.09).

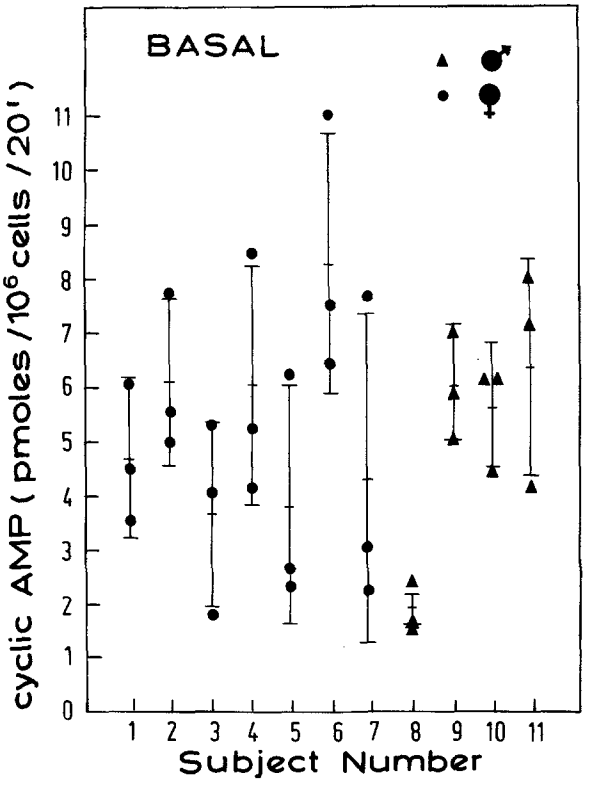

Figure 1. Basal cyclic AMP accumulation in intact human lymphocytes. Each point is the result of a single measurement on a individual subject at one time during the 30-day period. The vertical line represents the average of the three points ± SD.

Longitudinal measurements of forskolin-stimulated cyclic AMP accumulation in lymphocytes are shown in figure 2. A very significant difference was observed for cyclic AMP accumulation within the three samples from each subject compared to this variable between subjects (F = 5.83; DF = 10,20; p = 0.00041; msv between = 135.86 and msv within = 23.30). The significance levels for male and female subjects were: women (F = 10.04; DF = 6,12; p = 0.00043; msv between

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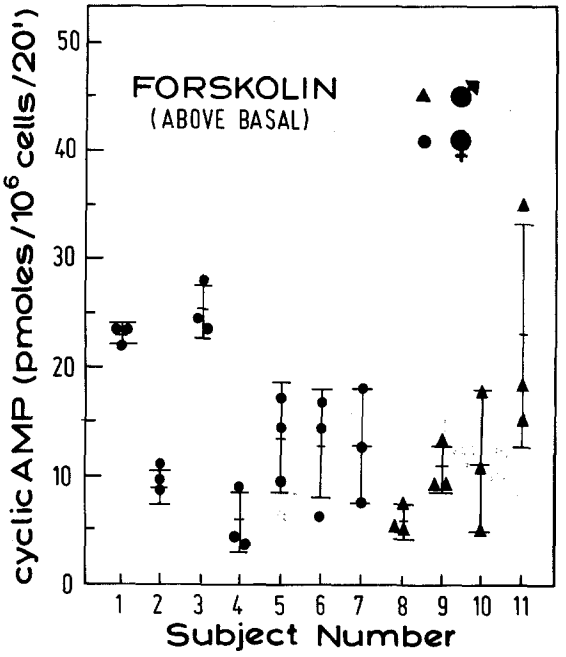

Figure 2. Forskolin-stimulated cyclic AMP accumulation in intact human lymphocytes. The concentration of forskolin was 60 μM.

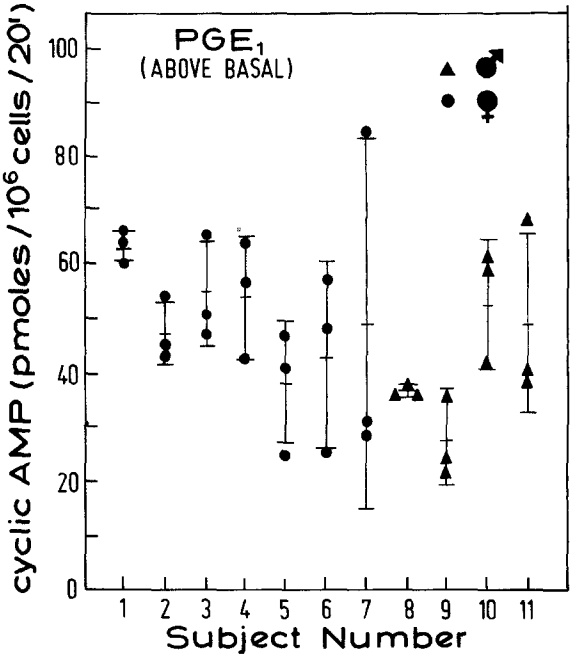

Figure 4. Prostaglandin E_1-stimulated cyclic AMP accumulation in intact human lymphocytes. The concentration of PGE_1 was 1 μM.

= 146.16 and msv within = 14.54), men (F = 3.47; DF = 3,6; p = 0.09; msv between = 149.70 and msv within = 43.13).

Longitudinal measurements of isoproterenol-stimulated cyclic AMP accumulation are shown in figure 3. No significant difference between cyclic AMP accumulation within the three samples from each subject and this variable between subjects was observed (F = 1.79; DF = 10,20; p = 0.128; msv between = 40.47 and msv within = 22.54). The significance levels for male and female subjects were: women (F = 1.22; DF = 6,12; p = 0.36; msv between = 39.75 and msv within = 32.40), men (F = 4.33; DF = 3,6; p = 0.06; msv between = 43.28 and within msv = 9.98). Similar results were observed for prostaglandin E_1-stimulated cyclic AMP accumulation (fig. 4). No significant difference between cyclic AMP accumulation within the three samples for each subject and this variable between subjects was observed (F = 2.07; DF = 10,20; p = 0.079; msv between = 34.48 and msv within = 16.65). The significance levels for male and female subjects were: men (F = 6.79; DF = 3,6; p = 0.023; msv between = 445.01 and msv within = 65.49), women (F = 0.809; DF = 6,12; p = 0.58; msv between = 210.63 and msv within = 260.29).

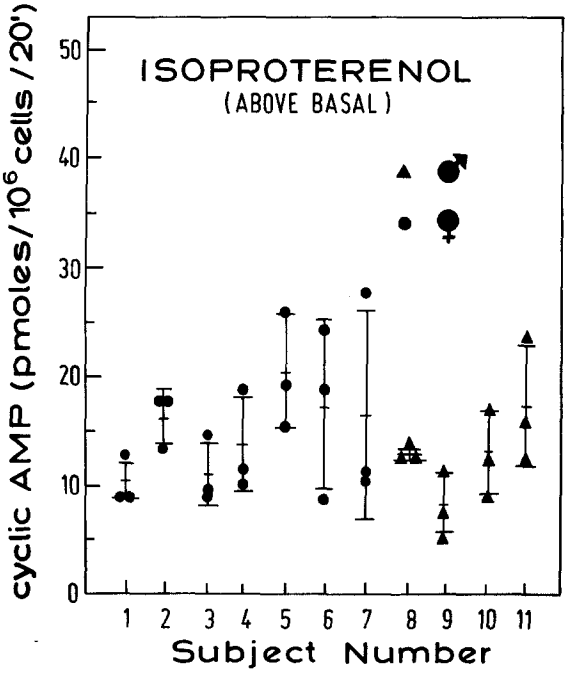

Figure 3. Isoproterenol-stimulated cyclic AMP accumulation in intact human lymphocytes. The concentration of isoproterenol was 10 μM.

In the present study the reliability of estimating basal, forskolin and hormone-stimulated cyclic AMP accumulation in intact lymphocytes was examined. Although lymphocyte adenylate cyclase activity has been studied extensively in a number of clinical investigations[2-11], little attention has been paid to the reproducibility of longitudinal measurements in human subjects. The results from the current study demonstrate a very significant reliability for forskolin-induced cyclic AMP accumulation over a one-month period in lymphocytes obtained from a group of 11 young, healthy subjects. These findings are not surprising since we have recently shown that individual differences in forskolin-stimulated adenylate cyclase activity in human lymphocytes is under a large degree of genetic control[22]. The higher the heritability the less likely a variable is to be influenced by random environmental factors. Such factors would tend to reduce the reliability of longitudinal measurements. Both the current investigation and our previous genetic study[22] suggest that forskolin-stimulated adenylate cyclase activity in lymphocytes may prove to be a valuable 'trait' characteristic in studies of human pathology.

Recent evidence suggests that forskolin has effects on the catalytic subunit as well as on input from the stimulatory (Ns) and inhibitory (Ni) guanine-nucleotide binding regulatory subunits[23,24]. Since forskolin can also activate adenylate cyclase in the absence of the N subunit, it is likely that forskolin acts primarily at a site on the catalytic subunit[24]. Forskolin stimulation of adenylate cyclase in intact lymphocytes, therefore, is a measure of intrinsic enzyme activity not mediated by hormone receptors. The high reliability of repeated measurements of fors-

kolin activity from individual subjects in lymphocytes is consistent with the role of the forskolin site in the receptor-linked adenylate cyclase system. Most physiological changes in adenylate cyclase activity are mediated through either up or down regulation of hormone-receptor number and less frequently through adjustments in activity of the catalytic subunit[6, 21, 25–27]. It should be noted, however, that recent evidence suggests that changes in receptor coupling due to changes in the proportion of Ni and Ns and the modification of Ns may also be important in regulation of adenylate cyclase[11].

Longitudinal measurement of basal cyclic AMP accumulation in intact lymphocytes obtained from individual subjects is not a very reliable measure. The difference between basal cyclic AMP accumulation within repeated samples from the same subject and this variable between subjects just attains statistical significance ($p = 0.049$). On the other hand, reproducibility of repeated measurements from the same individual was greater in male compared to female subjects. Similar results were obtained for isoproterenol and prostaglandin E_1-stimulated cyclic AMP accumulation. For these two receptor-stimulated activities, however, no difference between repeated samples from the same individual and lymphocytes from different individuals could be shown. However, for male subjects a significant difference in within subject and between subject samples could be shown for both isoproterenol and prostaglandin E_1 activities. Measurement of cyclic AMP accumulation in lymphocytes from men appears to be a more reliable parameter than from women.

In a recent report from this laboratory[22] the individual differences observed in levels of beta-adrenergic and prostaglandin receptor-stimulated cyclase activity in human lymphocytes were shown to be a 'state' characteristic reflecting environmental influences on receptor activity: a significant genetic contribution could not be demonstrated in the control of the levels of these two receptor activities in human lymphocytes. These results are not surprising since in lymphocytes both receptor number and receptor-linked adenylate cyclase activity respond rapidly to changes in hormone concentrations[6, 21, 25–27]. It is this rapid response to changes in environmental cues that most likely contributes to the failure in the current study to obtain reproducible measurements for these activities in lymphocytes obtained from the same individual.

The trend for measurements in male subjects to be more reliable suggests that hormonal influences, perhaps related to the female menstrual cycle, play a role in regulating beta-adrenergic and prostaglandin E_1 receptor activities in human lymphocytes. Such influences could markedly reduce the reliability of such studies when female subjects are participants. We are now investigating changes in hormone-stimulated cyclic AMP accumulation in lymphocytes obtained from women in different stages of the menstrual cycle and have observed a significant decline in isoproterenol-stimulated activity during the menses phase[28]. Finally it should be noted that the number of male subjects in the current study was small and further investigations are required before the reliability of hormonal-stimulated cyclic AMP measurements in lymphocytes from male subjects can be demonstrated unequivocally.

The purpose of the present study was to establish the usefulness of the lymphocyte model in studying the cyclic AMP second messenger amplification system in man using the 'usual' experimental protocols commonly employed in many clinical investigations. Most previous studies[2–11, 20, 21] have employed lymphocytes obtained from various subject groups without enforcing a stringent control of subject dietary intake or, when women were included, without sampling at a fixed time during the menstrual cycle. Without absolutely standardized methodology the present results suggest that only for forskolin-stimulated activity is the usual procedure adequate. When isoproterenol and prostaglandin E_1-stimulated cyclic AMP accumulation are measured in lymphocytes, undefined environmental influences can markedly affect hormone-stimulated cyclic AMP signal transduction in lymphocytes obtained from individual subjects. Of course, if sufficiently strong experimental intervention such as use of beta-adrenergic antagonists is employed, then changes in lymphocyte cyclic AMP second messenger signal transduction will reflect pharmacological treatment[29]. Similarly, if the effect of pathophysiological conditions are severe then lymphocyte hormone-stimulated adenylate cyclase activity will also reflect the disease state[2, 8]. However, more subtle changes in function may go undetected unless subject selection and clinical status are severely controlled.

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