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Lymphocyte Protein Kinase Activity in Cells from Young and Elderly Men and Women[1] (42203)

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Abstract. Protein kinase activity of lymphocytes isolated from human subjects was assayed using histone as substrate. The activity was stimulated about twofold by cyclic AMP and total enzyme activity, determined in the presence of cyclic AMP, was inhibited by 65% by the specific heat-stable inhibitor of cyclic AMP-dependent protein kinase. Histone phosphorylation was not stimulated by cyclic GMP in the presence of the inhibitor. Cyclic AMP-dependent protein kinase could be activated *in vitro* by incubating intact cells with isoproterenol or with forskolin and was reflected by a significant ($P < 0.05$) increase in the protein kinase activity ratio. In contrast to these well-characterized adenylate cyclase activators, incubating cells for up to 2 hr *in vitro* in the presence of the specific beta-blocker propranolol had no significant effect on the amount of cyclic AMP-dependent protein kinase that was in the activated state. When compared in subjects between the ages of 21 and 74 years, lymphocyte protein kinase activity was unaltered by age or gender. These results indicate that cyclic nucleotide-dependent protein kinase is of the cyclic AMP-dependent variety in the human lymphocyte. A low amount of the cyclic AMP-dependent activity (about 15%) is in the already activated state in freshly isolated cells, and this is not further reduced by incubation *in vitro* or by beta-blockade. In contrast to previously reported changes in the capacity to synthesize cyclic AMP, lymphocyte protein kinase is unaltered by gender or age in human subjects.

The most accessible β-adrenergic catecholamine target tissue in man is the circulating lymphocyte. Since the action of the β-adrenergic receptor is cyclic AMP-mediated in most target tissues, changes in lymphocyte cyclic AMP production associated with age and gender may reflect mechanisms that account for reduced catecholamine sensitivity in less accessible human tissues (1–4). In mammalian cells the intracellular effects of cyclic AMP are mediated in turn by activation of cyclic AMP-dependent protein kinase (CAMP-PK) so that this class of protein phosphorylating enzymes is an important part of the hormone-sensitive adenylate cyclase system in the human lymphocyte (5).

Differences in target tissue sensitivity and cyclic AMP production in man might be accompanied by concomitant changes in CAMP-PK. We characterized, therefore, CAMP-PK present in lymphocyte homogenates from men and women between the ages of 21 and 74 years, groups in which cyclic AMP synthesizing capacity has been reported to change (3, 4). The results suggest the extent to which differences in human lymphocyte cAMP-PK can be attributed to age or gender.

Materials and Methods. Lymphocytes were isolated from human subjects between the ages of 20 and 75 years who were judged to be disease free and unmedicated. The single-step centrifugation method using lymphocyte separation medium (Bionetics Laboratory Products, Kensington, Md.) was used as described previously (3) except all of the isolation procedures were carried out at 24°C. The isolated cells were resuspended in protein kinase homogenization buffer (0.01 *M* Na^+ phosphate buffer, pH 6.8; 0.001 *M* EDTA) at 4°C at a cell density of 25×10^6 cells/ml using a Teflon–glass homogenizer, or at a density of 2×10^6 cells/ml in RPMI 1640 containing 10% bovine calf serum for incubation of intact cells *in vitro*. The homogenates were used immediately for the determination of protein kinase activity as described below.

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The incubations *in vitro* were carried out in the presence of the additions and for the periods described in the text by shaking the intact cell suspensions at 37°C with continual gassing (95% O_2; 5% CO_2). The cell suspensions were chilled to 4°C at the end of the incubation period and the cells concentrated by centrifugation at 1000*g* for 10 min (4°C). The cell pellet was rinsed and the cells resuspended in the protein kinase homogenization buffer as described above.

Protein kinase activity was determined by adding 0.02 ml of the cell homogenate (the equivalent of 500,000 cells) to 0.13 ml of the assay buffer which was 0.2 *M* Na^+ phosphate (pH 6.5), 0.01 *M* $MgCl_2$, 3.75×10^{-7} mole ATP and 0.25–0.30 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, Mass.), and a saturating concentration (25–100 μg) of histone (Type IIAS, Sigma, St. Louis, Mo.). The temperature of the mixture was simultaneously shifted from 4 to 37°C. The reaction was stopped by the addition of 1.5 ml of ice-cold 10% trichloroacetic acid (TCA) after 12 min. After a minimum of 30 min at 4°C the acid-insoluble precipitates were collected by filtration onto glass fiber filters which were then washed four times with 2 ml of ice-cold 5% TCA. The filters were dried and counted in a scintillation cocktail without surfactant.

Tubes containing the complete reaction mixture (including homogenate) except histone were included and processed in an identical manner for subsequent correction by subtraction of histone-independent radiophosphorylation. Using these methods, background phosphorylation in the absence of added histone was <5% of histone phosphorylation.

Results. Cyclic nucleotide-dependent lymphocyte protein kinase activity was characterized using the heat-stable specific inhibitor of CAMP-PK. Figure 1 shows that lymphocyte protein kinase activity was stimulated about twofold by the addition of cyclic AMP and much less by cyclic GMP. The specific inhibitor (PKI) depressed total protein kinase activity (determined by assaying lymphocyte homogenates in the presence of cyclic AMP) in a dose-dependent manner up to about 30 μg of the inhibitor above which there was no significant ($P > 0.05$) additional inhibition. In 12 different lymphocyte homogenates, a saturating concentration of PKI (40 μg) inhibited total protein kinase activity by 65 ± 3% (mean ± SEM). Cyclic GMP failed to stimulate lymphocyte protein kinase in the presence of PKI, indicating that all of the cyclic nucleotide-dependent protein kinase activity was PKI-sensitive (~65%) and represented CAMP-PK (6). About 35% of the total activity in lymphocytes was due, therefore, to cyclic nucleotide-independent protein kinase.

There were no significant differences ($P > 0.05$) in total protein kinase activity of lymphocytes from men or from women in any age group (not shown). Similarly, the distribution of activated compared to unactivated protein kinase as reflected by the protein kinase activity ratio (activity in the absence of cyclic AMP divided by the total activity determined in the presence of cyclic AMP) was unaffected by age or by gender (Fig. 2). Whether lymphocyte CAMP-PK responded to increased cyclic AMP production was determined *in vitro.* Lymphocyte CAMP-PK was rapidly activated *in vitro* by incubating cells for 5 min with the specific β-adrenergic agonist isoproterenol or with the labdane diterpene forskolin which stimulates cyclic AMP production in a non-receptor mediated fashion (7). Activation under these conditions was indicated by a significant increase ($P < 0.05$) in the protein kinase activity ratio which reflected an increase in enzyme activity assayed in the absence but not in the presence of cyclic AMP (Fig. 3). These results, obtained *in vitro,* suggested stimulation of cyclic AMP synthesis by endogenous agonists *in vivo* might account for some of the observed properties of protein kinase in the isolated lymphocytes. Partial activation *in vivo* might obscure differences due to age or gender, for instance.

β-Adrenergic catecholamine exposure both *in vivo* and *in vitro* has also been shown to reduce lymphocyte sensitivity to agonists by inducing a desensitized state (8). We investigated, therefore, whether some properties of CAMP-PK might be due to a desensitized state caused by endogenous catecholamines. In order to determine whether any desensitization might be rapidly reversed, lymphocytes were incubated for up to 2 hr in the presence of the specific β-adrenergic antagonist propranolol

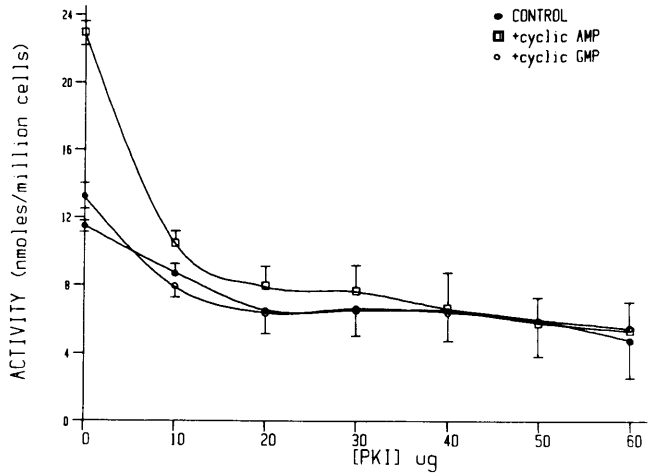

FIG. 1. Sensitivity of human lymphocyte protein kinase activity to the heat-stable specific inhibitor of cyclic AMP-dependent protein kinase. Lymphocyte homogenates were assayed according to the procedure described under Materials and Methods and in the presence of the indicated concentrations of the specific inhibitor (PKI) in the absence (control) or presence of the indicated cyclic nucleotides (2 μM). The values represent the means ± SEM of results obtained with 4–6 separate preparations.

(Fig. 4). Beta-blockade carried out *in vitro* had no significant ($P > 0.05$) effect on protein kinase activity or its stimulability by cyclic AMP in cells from either young (<50 years) or elderly (>50 years) subjects (Table I).

Discussion. Cyclic nucleotide-dependent protein kinase activity in crude homogenates of freshly isolated human lymphocytes was the cyclic AMP-dependent variety (CAMP-PK) and could not be significantly stimulated by cyclic GMP in the presence of PKI. About 65% of the total activity in the homogenates was inhibited by PKI, indicating that a greater fraction of lymphocyte protein kinase than has been reported previously is CAMP-PK (9–11). This difference might be accounted for by the

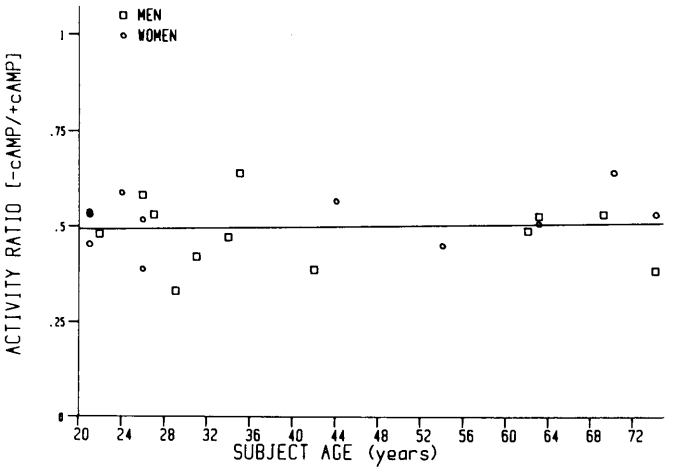

FIG. 2. Effect of subject age and gender on the lymphocyte protein kinase activity ratio. Freshly isolated cells from male or female subjects of the ages indicated were assayed as described under Materials and Methods in the absence or presence of 2 μM cyclic AMP for subsequent calculation of the protein kinase activity ratio. The line, determined by regression analysis, was not significantly different from zero ($P > 0.05$) and did not differ significantly ($P > 0.05$) between the two groups.

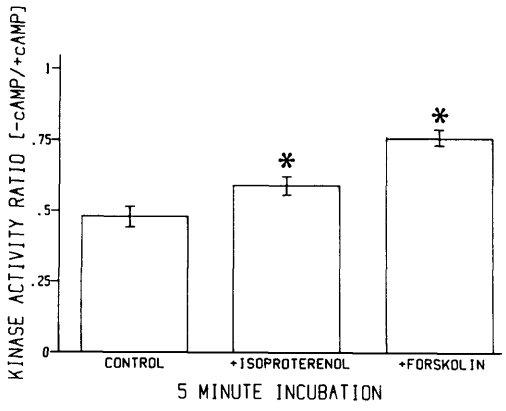

FIG. 3. Activation of lymphocyte CAMP-PK *in vitro*. Intact cells were incubated in the absence (control) or presence of 100 μM isoproterenol or forskolin for 5 min under the conditions described under Materials and Methods before centrifugal washing and resuspension for homogenization. Homogenates were assayed for enzyme activity in the absence and the presence of 2 μM cyclic AMP and the results were expressed as the protein kinase activity ratio. The values are the means ± SEM of results obtained with 5 separate preparations. *, Significantly ($P < 0.05$) greater than the control value.

previous characterization of histone kinase activity in fractionated lymphocytes. We characterized the activity in crude homogenates since protein kinase activation leads to its redistribution by translocation in several tissues and differences in intracellular distribution might be confused, therefore, with age- or sex-related differences (12–14). In the human lymphocyte, for instance, there is evidence that cyclic AMP as well as protein kinase is subcellularly compartmentalized (15, 16).

Cyclic AMP stimulated lymphocyte protein kinase about twofold, and in 23 separate samples 50 ± 2% (mean ± SEM) of the total activity (determined in the presence of cyclic AMP) was cyclic AMP independent and represented, therefore, a mixture of noncyclic nucleotide-dependent protein kinase and CAMP-PK in an already activated state. PKI Inhibited protein kinase activity to 35 ± 3% of that determined in the presence of cyclic AMP (total activity), suggesting that about 35% of lymphocyte protein kinase was of the noncyclic nucleotide-dependent variety. Only about 15% of CAMP-PK was therefore in an already activated state in the intact cells. The distribution of CAMP-PK between its preactivated and unactivated states may represent the "basal" state of the lymphocyte activity *in vivo* since the enzyme could be rapidly activated *in vitro* by exposure to isoproterenol or forskolin, drugs that elevate cyclic AMP levels in broken cell as well as intact lymphocyte preparations (3, 17).

Cyclic AMP production by lymphocytes is associated principally with T-cell growth and

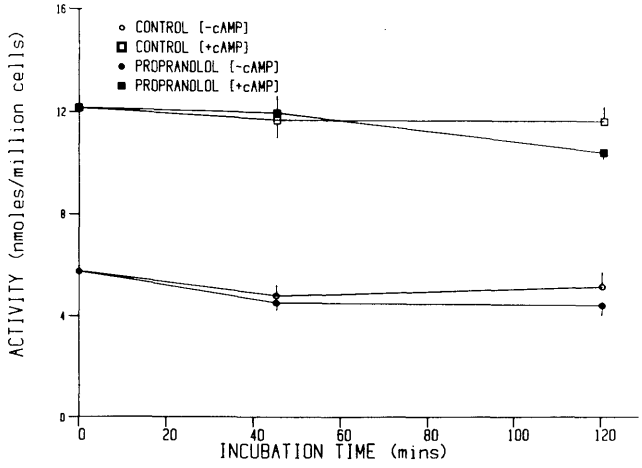

FIG. 4. Effect of *in vitro* beta-blockade on lymphocyte protein kinase activity. Freshly isolated intact cells were incubated for the periods indicated in the absence (control) or presence of 1 nM (−)-propranolol as described under Materials and Methods prior to centrifugal washing and resuspension for homogenization. Homogenates were assayed in the absence (−cAMP) or presence (+cAMP) of 2 μM cyclic AMP as described. The results are the means ± SEM of those obtained with 8 separate preparations.

TABLE I. EFFECT OF PROPANOLOL ON LYMPHOCYTE PROTEIN KINASE ACTIVITY

Age group (years)	Control		+ 1 nM Propranolol		
	Protein kinase activity[a]	Activity ratio[b]	Protein kinase activity[a]	Activity ratio[b]	P^c
21–42 (N = 8)	12.64 ± 1.57	0.48 ± 0.03	11.74 ± 1.82	0.44 ± 0.05	>0.05
54–74 (N = 7)	12.12 ± 0.72	0.52 ± 0.03	10.77 ± 0.93	0.47 ± 0.04	>0.05
P^d	>0.05	>0.05	>0.05	>0.05	—

Note. Cells were assayed before (control) and after a 45-min incubation with the indicated concentration of propranolol in the presence and absence of cyclic AMP. The results are the means ± SEM of the number of different samples indicated in each group.

[a] Total enzyme activity in nanomoles per million cells determined in the presence of cyclic AMP.

[b] Activity − cyclic AMP/activity + cyclic AMP.

[c] Difference due to propranolol treatment by unpaired t test.

[d] Difference due to age by unpaired t test.

differentiation in mammals including man (11, 18). Differences in cyclic AMP producing capacity could contribute, therefore, to differences in immune function due to age and gender since these also effect lymphocyte adenylate cyclase activity (19, 20). However, the role of protein kinase activation and the phosphorylation of endogenous lymphocyte proteins is complex. There appear to be varieties of CAMP-PK and phosphoproteins specifically associated with both growth and differentiation in the lymphocyte. Although the cyclic AMP-dependent phosphorylation of several different lymphocyte proteins increased or decreased according to the proliferative stage, CAMP-PK was markedly reduced in dedifferentiated lymphocytic leukemia cells compared to normal circulating cells arrested at the same nonproliferative stage (11, 16). Therefore, it appears that although age- or gender-related differences in protein kinase do not account for differences in lymphocyte function, protein phosphorylation does change in response to altered cell function mediated by cyclic AMP production.

While the role of cyclic AMP in lymphocyte differentiation and proliferation is complex, the importance of increased cyclic AMP production in response to agonists such as isoproterenol, which are not generally associated with growth or differentiation, is even more obscure. Previous investigations suggested that although isoproterenol and series E prostaglandins stimulated large increases in cyclic AMP production by human lymphocytes, they had no effect on endogenous substrate phosphorylation (18). In contrast, we found that isoproterenol exposure activated CAMP-PK and significantly increased the protein kinase activity ratio. These results demonstrate that β-adrenergic receptors are functionally coupled with endogenous substrate phosphorylation and metabolic regulation in the lymphocyte even though the specific nature of these remains ill defined.

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