Early Stages in the Mechanism of Action of Glucocorticoids on Human Platelets. Effect of Hydrocortisone on Intracellular cAMP and Ca^{2+} Content

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Changes in basal and stimulated levels of cAMP and calcium induced by hydrocortisone in a wide range of concentration (0.1-25 μM) are studies in a suspension of washed human platelets. The effects of hydrocortisone on the activity of preparations modulating various stages of the adenylate cyclase system (forskolin, adenosine, adrenaline, and 3-isobutyl-1-methylxanthine) are compared. Platelets are stimulated with collagen, platelet activating factor, and thapsigargin. Hydrocortisone in different concentrations acts as both activator and inhibitor of calcium metabolism in platelets.

Key Words: *platelets; calcium; cAMP; hydrocortisone; thapsigargin; inhibitors of platelet activation*

The rise in the concentration of free calcium ions in the cytoplasm $[Ca^{2+}]_{cyt}$ is the trigger mechanism of platelet activation. The rise of $[Ca^{2+}]_{cyt}$ results in activation of Ca^{2+}-dependent membrane-bound enzymes, phospholipases A_2 and C, stimulation of cell contractile apparatus followed by secretion release from the secretory granules, and expression of adhesive protein receptors on the platelet surface [11]. The platelet Ca-response involves the calcium-phosphoinositide system and three types of Ca channels in the plasma membrane: Ca^{2+} channel of P_{2x}-purine receptor (activated by ADP and ATP), Ca^{2+} channels regulated by second messenger (calcium ions, inositol-1,4,5-tris-phosphate); and potential-independent G-protein-operated Ca channels (activated by α-subunit of G-protein) [1].

Adenylate cyclase activators inhibit the rise of $[Ca^{2+}]_{cyt}$ and usually suppress Ca^{2+}-dependent processes in platelets [8]. Similar effects are exhibited by the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and membrane-permeable dibutyryl derivative of cAMP (dbcAMP) [6].

Hydrocortisone (HC) inhibits platelet activity [2]. In concentrations of 1-25 μM it inhibits collagen-induced platelet aggregation and potentiates adenosine-induced platelets disaggregation. For evaluation of molecular mechanisms of HC effect on platelet activity we examined the effect of HC on basal and agonist-stimulated levels of cAMP and Ca^{2+}.

MATERIALS AND METHODS

Preparation of washed human platelet suspension, Fura 2-AM (Calbiochem) loading, and calculation of free calcium concentration in cells were described previously [4]. The content of cAMP was determined by radioligand assay using Amersham kits. The following reagents were used: platelet activating factor (Sigma), adrenaline, collagen and adenosine (Serva), hydrocortisone (Merck), forskolin and thapsigargin (RBI).

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The data were processed statistically using Pharmacological Basic Statistics software. The confidence intervals of the experimental values and significance of differences were evaluated by Student's t test at p=0.05.

RESULTS

The basal cAMP level in resting platelets was 6.9±1.2 pmol/10^9 cells (n=14). At 0.1-1.0 μM HC had no effect and at 2-25 μM elevated basal level of cAMP in a dose-dependent manner. This elevation became significant at HC concentrations equal or above 5 μM (10.6±1.3 pmol/10^9 cells, n=5). In the presence 25 μM HC, the content of cAMP attained its maximum (14.7±2.5 pmol/10^9 cells, n=5) surpassing the baseline value by 105% (10-min incubation at 37°C in all experiments). In the next experimental series, the ability of HC to modulate cell cAMP response to platelet adenylate cyclase activators (adenosine and forskolin) and inhibitors (adrenaline) was studied.

The direct activator of cAMP synthesis forskolin (20 μM) induced more than 7-fold increase in the cell cAMP content (47.1±8.9 pmol/10^9 cells, n=4). The receptor-dependent elevation of cAMP induced by adenosine (mediated through A_2-adenosine receptors) was less pronounced: 14.4±2.6 and 25.7±3.7 pmol/10^9 cells (n=5), for adenosine concentrations 1 and 3 μM, respectively. HC (0.1-2.0 μM) potentiates the adenosine-induced rise of cAMP in a dose-dependent manner and has no effect on the forskolin-induced changes in the content of cAMP.

In combined application of 3-isobutyl-1-methylxanthine (100 μM) and HC (25 μM), we observed a simple summation of the effect of these preparations on cAMP level in platelets. This probably attests to the absence of direct effect of HC on cAMP phosphodiesterase activity.

It is known that adrenaline effectively inhibits platelet adenylate cyclase via α_2-adrenoreceptors [7]. In our experiments, adrenaline (20 μM) reduced basal content of cAMP by 24%; HC (0.5-2.0 μM) in combined application diminished the inhibiting effect of adrenaline. For instance, in the presence of 2 μM HC the adrenaline-induced drop of cAMP content constituted no more than 15% (p<0.05). These findings support our assumption on the participation of G_i-proteins in realization of nongenomic effects of glucocorticoids [3]. The effects of higher doses of HC on cell response to adrenaline are difficult to assess due to intrinsic stimulating effect of HC on cAMP level.

Measurements of $[Ca^{2+}]_{cyt}$ revealed a considerable decrease in the basal calcium level (85±14 nM, n=7) caused by high concentration of HC (above 10 μM). For evaluation of the effect of HC on the stimulated rise of $[Ca^{2+}]_{cyt}$ we choose platelet activators acting through diverse mechanisms: platelet activating factor (100 nM) and collagen (0.75 mg/ml). Collagen predominantly activates calcium entry into platelets, while platelet activating factor primarily mobilizes calcium from intracellular stores [10].

Collagen-induced rise of $[Ca^{2+}]_{cyt}$ (ΔCa^{2+} calculated as the difference between calcium levels in the presence and absence of inductor) attains 241± 2.8 nM (n=4). Platelet activating factor induces a greater increase in $[Ca^{2+}]_{cyt}$: 322±31 nM (n=4). HC (0.5-5.0 mM) inhibited in a dose-dependent manner the rise of $[Ca^{2+}]_{cyt}$ induced by both activators; however, the inhibiting effect of HC against the collagen-induced Ca-response was more pronounced, which implies a selective effect of HC on Ca^{2+} transport through the platelet plasma membrane. Comparison of the Ca-blocking effect of HC in the medium containing 1 mM $CaCl_2$ and in a calcium-free buffer (0.1 mM EGTA) indirectly confirmed the assumption that HC in the studied concentrations primarily inhibits calcium entry into platelets and does not affect its mobilization from cell stores. To clarify the mechanism of the effect of HC, a selective inhibitor of Ca^{2+} ATPase thapsigargin [9] was used. In cells treated with 1 μM thapsigargin, which induces Ca^{2+} release from intracellular stores and prevents its accumulation, no effect of HC on $[Ca^{2+}]_{cyt}$ was detected.

In the presence of higher concentrations of HC (5-25 μM) in the incubation medium we observed more pronounced changes in the inductor-dependent rise of $[Ca^{2+}]_{cyt}$. Under these conditions HC inhibited both the receptor-dependent calcium entry and Ca^{2+} mobilization in platelets. This effect is apparently mediated through cAMP, since activators of adenylate cyclase (adenosine, prostacyclin, and prostaglandin E_2) also inhibit the rise of $[Ca^{2+}]_{cyt}$ in response to inductors of platelet aggregation [1].

Thus, it can be concluded that HC in different concentrations may act as an inhibitor and activator of calcium metabolism in platelets.

HC in concentrations of 0.1-1.0 μM, i.e., within the physiological concentrations of glucocorticoid hormones in human blood has no effect on basal cAMP and Ca^{2+} levels, but potentiates the agonist-induced rise of cAMP concentration in platelets and indirectly inhibits the cell calcium response. In concentrations of 2.0-5.0 μM (mean therapeutic concentration of glucocorticoids in the plasma) HC has no effect on the basal levels of cAMP and Ca^{2+} but reduces the agonist-induced calcium entry most likely through G-operated Ca-channels in the platelet plasma membrane.

In high concentrations (5-10 μM, corresponding to high pharmacological doses of glucocorticoids) HC elevates basal cAMP level suppressing the effector stages of the Ca-response activated by products of phosphatidylinositol tris-phosphate hydrolysis.

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Comparative Study of Na-Blocking Properties of Antiarrhythmic Drugs on Isolated Rat Cardiomyocytes

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The effects of the antiarrhythmic preparations lidocaine, flecainide, and rihlocaine on sodium concentration in cardiomyocyte cytoplasm are studied using the Na-sensitive fluorescent probe SBFI. The Na-blocking effect of lidocaine and rihlocaine depends on the frequency of electrical stimulation of cardiomyocytes (0.2-1.0 Hz). The data suggest the possibility of *in vitro* testing of novel antiarrhythmic drugs.

Key Words: *lidocaine; flecainide; hypoxia; sodium channel blockers; antiarrhythmic drugs; cardiomyocytes*

According to modern classification of antiarrhythmic drugs, sodium channel blockers (group I) are divided into three subgroups (Ia, Ib, and Ic) [8]. This division is made on the basis of the molecular mechanisms of interaction between antiarrhythmic agents class I (AAI) and voltage-dependent sodium channels (Na^+ channels) in the plasma membrane of electroexcitable cells [4]. There are three functional states of these channels: open, closed, or inactivated (refractory). AAI bind only to open or refractory channels and therefore block only **functioning** Na^+ channels; moreover, the higher heart rate, the stronger pharmacological effect of AAI [4], especially of agents of Ib subgroup (AAIb). These drugs (lidocaine and phenytoin) rapidly associate and dissociate with Na^+ channel, and their effect is completely reversible. Antiarrhythmic agents of subgroup Ic (flecainide, encainide, etc.) are characterized by a lower rate of dissociation and therefore exert a slower but prolonged effect (low-reversible). Apart from their Na^+-blocking activity, AAIc inhibit K^+ current through Ca^{2+}-insensitive K^+ channels [7]. AAIa such as quinidine and procainamide occupy an intermediate position between AAIb and AAIc.

The subgroups of AAI determine the peculiarities of clinical application of the given preparation and

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