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cAMP reduces the affinity of Ca^{2+}-triggered secretion in platelets

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Prostacyclin and other related compounds known to increase intracellular cAMP levels inhibit platelet responses. The mechanisms involved are only partially known, especially those concerning the complex relations between Ca^{2+} and cAMP as opposite intracellular mediators. Here, we have investigated aggregation and secretion in quin2-loaded platelets under conditions in which Ca^{2+} and cAMP are the only intracellular mediators. Our results show that cAMP inhibits aggregation and secretion in ionophore-treated cell without modifying their intracellular Ca^{2+} levels. This result suggests that the inhibition takes place on some intracellular target for Ca^{2+}.

cyclic AMP; Ionomycin; Secretion; Platelet; intracellular Ca^{2+}

1. INTRODUCTION

The rise of cytoplasmic Ca^{2+} levels is probably the main trigger of platelet exocytotic secretion even though other messengers such as thromboxanes and DAG are also involved in physiological responses [1]. cAMP acts as a negative modulator by inhibiting key regulatory enzymes like phospholipases C and A_2, cyclo-oxygenase and thromboxane synthetase [2]. However, the effect of cAMP on intracellular Ca^{2+} homeostasis remains not well defined.

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Abbreviations: IBMX, isobutylmethylxanthine; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine); TPA, phorbol ester; OAG, oleoylacetylglycerol; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide; DAG, 1,2-diacylglycerol

We report here the results of experiments in which the Ca^{2+} ionophore ionomycin was used to activate aggregation and exocytotic secretion in human platelets treated with aspirin. Under these conditions Ca^{2+} is the only possible intracellular trigger, since the thromboxane pathway has been inhibited and phosphatidylinositol turnover is negligible in ionophore-treated cells [3]. Forskolin, an agent known to raise intracellular cAMP levels by direct stimulation of adenylate cyclase [4], was used to determine whether cAMP exerts its action by modifying the cytoplasmic Ca^{2+} rise induced by the ionophore treatment or by affecting subsequent steps of the Ca^{2+}-transducing mechanism.

2. EXPERIMENTAL

Platelet-rich plasma (PRP) was obtained by centrifugation of freshly drawn blood, anticoagulated with acid-citrate-dextrose (ACD), for 5 min at $800 \times g$ [5]. Cells were loaded with the fluorescent Ca^{2+} indicator quin2 by incubation of PRP with 15 μM quin2 acetoxy methyl ester for 30 min at 37°C. The platelets were then centrifuged at 350 ×

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g for 20 min and resuspended in a medium containing (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 10 Na-Hepes, pH 7.40. Thromboxane synthesis was blocked by incubating this suspension for 10 min at 37°C in the presence of 100 μM aspirin.

Aggregation was followed in a Payton aggregometer under continuous stirring [6]. Cells were first incubated for 1 min in medium containing 1 mM Ca^{2+}. Ionophore was then added and, after a fixed period of 3 min, samples of the cell suspension were centrifuged and the secreted ATP was measured in the supernatant solution [7]. Quin2 fluorescence was monitored in parallel experiments in a Perkin Elmer 204 spectrofluorimeter with monochromators set at 340 nm excitation and 500 nm emission. Intracellular Ca^{2+} values were calculated using the general formula:

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$$

A value of 116 nM was used for K_d [8]. F_{max} was obtained by adding to the cell suspension 400 nM ionomycin, a concentration known to give maximal effect. F_{min} was calculated as one-sixth of the difference between F_{max} and cell autofluorescence, which was estimated after addition of 2 mM $MnCl_2$ to quench the quin2 fluorescence [9]. The final concentration of quin2 in the cell water was always between 1 and 2 mM.

Intracellular cAMP levels were raised by a 2 min incubation with 20 μM forskolin plus 20 μM IBMX, a stimulator of adenylate cyclase and an inhibitor of phosphodiesterase, respectively. cAMP levels, measured by a specific radioimmunoassay (Amersham), were increased by this treatment from 1.79 ± 0.61 to 50.80 ± 10.79 pmol/10^8 cells (mean ± SE). It was also checked that the treatments with forskolin and ionophore did not produce non-specific membrane permeabilization, as shown by the fact that over 90% of LDH activity (Boehringer Mannheim Monotest) was retained by treated cells by the end of the incubation period.

All chemicals were obtained from Sigma except forskolin (Calbiochem), quin2 (a kind gift from Dr T.J. Rink, Smith Kline-French, London) and ionomycin (a personal gift from Dr V.L. Lew, Physiological Laboratory, Cambridge). All drugs were added to cell suspensions from concentrated solutions in ethanol (ionomycin, forskolin and IBMX), DMSO (quin2 acetoxymethyl ester) or buffer. The final concentration of organic solvent was always below 0.5%.

3. RESULTS AND DISCUSSION

Fig.1 shows simultaneous measurements of absorbance (A), ATP release (B) and fluorescence of quin2-loaded cells (C), in either control (top) or forskolin-treated cells (bottom). Forskolin treatment did not change the intracellular resting Ca^{2+} level, which was maintained around 70 nM under both conditions (fig.1C). This result suggests that

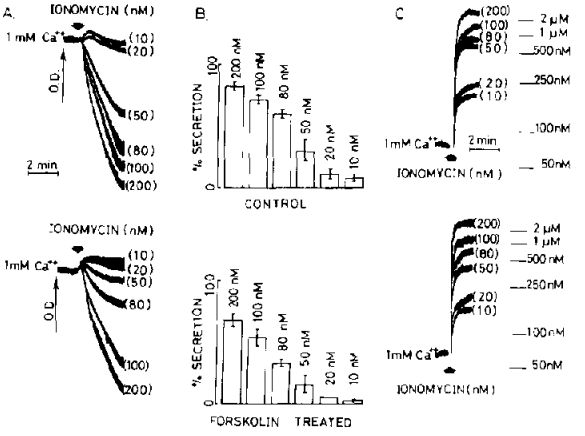

Fig.1. (A) Two sets of absorbance records in quin2-loaded platelets suspended in the usual medium containing 100 μM aspirin and 1 mM $CaCl_2$, in control cells (top) or 2 min after addition of 20 μM forskolin plus 20 μM IBMX (bottom). Experiments were performed at 37°C and under continuous stirring. One typical experiment representative of 3 similar ones is shown. (B) Histogram of means ± SE (3 experiments) of secreted ATP, measured in samples taken from the aggregometer cuvette whose records are shown in panel A. The values are expressed as a percentage of the maximal releasable pool, which was estimated from the response to 0.5 U/ml thrombin. Secretion of serotonin was also measured in a single experiment which gave similar results to those shown in this panel. (C) Two sets of records showing changes in the fluorescence of quin2-loaded cells. Measurements were preformed in parallel with the records of absorbance shown in panel A, and under the same experimental conditions. Calibration of the signal was made as specified in section 2. One typical experiment representative of 3 similar ones is shown.

cAMP does not lower the resting intracellular Ca^{2+} concentration by stimulation of mechanisms such as Ca^{2+} extrusion or Ca^{2+} sequestration in intracellular compartments, as proposed in [10]. Increasing concentrations of the Ca^{2+} ionophore ionomycin produced cells with progressively increased levels of Ca^{2+} which were maintained at a steady level for at least 3 min after stimulation. The recordings of quin2 fluorescence after six different ionomycin additions to either control or forskolin-treated cells were practically identical under both conditions, indicating that, at least during the period under study, elevated intracellular cAMP levels did not interfere with Ca^{2+} homeostasis. This result contrasts with previous reports, in which forskolin treatment completely abolished the intracellular Ca^{2+} rise induced by thrombin, collagen [11] or PAF [12]. This difference suggests that cAMP affects only the Ca^{2+} entry and release mediated by receptor-operated mechanisms and not by an artificial increase of Ca^{2+} permeability.

Aggregation and secretion of forskolin-treated cells were consistently lower than those of control cells (fig.1A,B). Since, as discussed before, Ca^{2+} levels were similar under both conditions, the inhibition could be interpreted as meaning that cAMP interferes with the generation of other intracellular mediators. DAG or thromboxanes would be likely candidates but, as mentioned in section 1, they play a negligible role under our experimental conditions. An alternative explanation would be that cAMP interferes with the action of Ca^{2+} on the secretion and aggregation machinery.

In fig.2 we have plotted the percentage of secretory effect vs intracellular Ca^{2+} levels in control and forskolin-treated cells. cAMP decreased the effect obtained at the highest intracellular Ca^{2+} concentration tested (~3 μM). On the other hand, the E_{50} value for Ca^{2+} was increased in the forskolin-treated preparation (900 vs 700 nM in the corresponding controls). These results are in apparent contrast with previous work [13], in which 20 μM cAMP did not affect the Ca^{2+} dose-response curves for serotonin secretion in high-voltage-permeabilized cells. However, the same authors have reported that 100 μM cAMP inhibited serotonin secretion induced by TPA or OAG, two direct activators of protein kinase C [14], in the same preparation [15]. In the light of the above findings, our results could be interpreted as indicating that, in our intact preparation, intracellular cAMP acts by lowering the Ca^{2+} affinity of protein kinase C, a key enzyme in the transduction of the secretory message [14]. Other possible actions responsible for the decrease in maximal effects of Ca^{2+} on ATP secretion cannot be discounted.

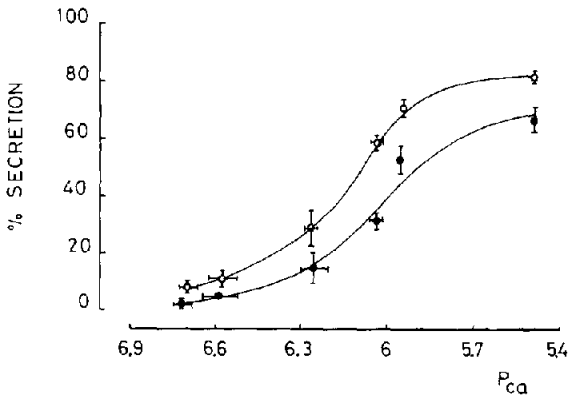

Fig.2. Secretion of ATP by quin2-loaded platelets as a function of intracellular Ca^{2+} concentration in control (○) and forskolin-treated cells (●). Note the logarithmic scale for Ca^{2+} concentration. Experimental conditions as in fig.1. Means ± SE of 3 experiments are shown.

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