Stimulation by serotonin of 40 kDa and 20 kDa protein phosphorylation in human platelets

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In human platelets, serotonin is known to induce a shape change followed by (reversible) aggregation. Recently, it was found that the amine triggers the elevation of cytosolic free calcium and activates phospholipase C. On stimulation of human platelets with serotonin we found an immediate increase in protein kinase C activity, phosphorylating its 40 kDa substrate protein. A 20 kDa protein, most likely the myosin light chain, was phosphorylated to the same extent. Ketanserin, a highly selective serotonin-S_2 antagonist inhibited both phosphorylation processes at subnanomolar concentrations.

Serotonin-S_2 receptor *Protein phosphorylation* *Platelet* *Ketanserin*

1. INTRODUCTION

In human platelets, serotonin induces a shape change followed by (reversible) aggregation [1]. When the cells are prelabelled with [^{32}P]orthophosphate, the addition of the amine results in a marked increase in ^{32}P incorporation in PA (here, [2]). The potency of several drugs to antagonize this PA-labelling was found to be directly related to their potency to inhibit platelet aggregation and to their binding affinities for serotonin-S_2 receptor sites [2]. It was suggested that part of the signal transducing system, which follows serotonin-S_2 receptor activation, involves phospholipase C catalyzed inositide breakdown (PI response) yielding diacylglycerol (DAG) which is subsequently phosphorylated to PA [2].

Authors in [3,4] found that the stimulus-evoked formation of DAG might serve as a signal for transmembrane control of protein phosphorylation through the activation of the protein kinase C (review [3,4]). In platelets this enzyme was found to phosphorylate a 40 kDa protein after stimulation with thrombin [5], collagen [5,6], platelet-activating factors [7,8] and arachidonic acid [9]. Thrombin and collagen also induce phosphorylation of a 20 kDa protein [6,10] that has been identified as the myosin light chain [10]. A specific Ca-calmodulin-dependent protein kinase was proposed to be responsible for this phosphorylation [10]. Here we investigated whether in the intact platelet both the endogenous substrate for the protein kinase C, and the myosin light chain kinase, are phosphorylated as a consequence of serotonin-S_2 receptor activation.

Abbreviations: PA, phosphatidic acid; DAG, diacylglycerol

2. EXPERIMENTAL

Human venous blood was collected from healthy volunteers in 0.2 vol. anticoagulant buffer containing 1.3% citric acid, 2.5% sodium acetate and 2% dextrose.

Platelets were prepared as in [11], except for the buffers. Washing buffer contained 25 mM Hepes (pH 7.2), 125 mM NaCl, 10 mM glucose, 1 mM EGTA and 1 mg/ml BSA. Final buffer consisted of 25 mM Hepes (pH 7.5), 125 mM NaCl, 2.7 mM KCl, 5.6 mM $MgSO_4$, 10 mM glucose, 0.1 mM EGTA, 1 mg/ml BSA. After addition of carrier free [^{32}P]orthophosphate (250 μCi/ml platelets), the platelet suspension was incubated at 37°C for

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60 min. Cells were stimulated with serotonin creatinine sulfate monohydrate (Janssen Chimica). Samples (150 μl: 4×10^8 cells) were taken at the time indicated and immediately transferred in 4.5 ml $CHCl_3/CH_3OH/0.45$ N HCl (20:40:13, by vol.). Two phases were obtained after the addition of 1.25 ml $CHCl_3$ and 1.25 ml 0.1 N HCl. After centrifugation the organic phase was washed with 2.5 ml $CH_3OH/0.2$ N HCl (1:1) and recentrifuged. The organic phase was evaporated under N_2, applied to silica gel 60 precoated plastic sheets (E. Merck, Darmstadt, FRG) and eluted in $CHCl_3/CH_3OH/20\%$ methylamine (60:35:10). After autoradiography on Kodak X-Omat R films, phosphatidic acid was located on the sheets and cut out. Radioactivity was quantitated by liquid scintillation spectrometry.

For protein analysis, 80 μl samples (2.1×10^8 cells) were quenched in a 5 times concentrated sample buffer [12] and incubated at 90°C for 5 min. Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% gel using the Laemmli buffers [12]. Gels were stained with Coomassie brilliant blue, dried and subjected to autoradiography. Areas corresponding to the 40 and 20 kDa proteins were cut out and radioactivity was determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

On addition of serotonin to human platelets prelabelled with [^{32}P]orthophosphate, maximal increase in [^{32}P]PA was observed at $2–4 \times 10^{-6}$ M amine [2]. Using 4×10^{-6} M serotonin we found an increase in ^{32}P-labelled 20 kDa and 40 kDa proteins (fig.1). Both increases reach their maximal phosphorylation, respectively, within 10 and 20 s after stimulation after which a decrease to approx. 20% is observed within 1 and 2 min. Maximal 40 kDa protein phosphorylation precedes the maximum for [^{32}P]PA formation which is consistent with the idea that DAG, the metabolic precursor of this phospholipid, is the possible activator of the protein kinase C.

The formation of ^{32}P-labelled 40 kDa and 20 kDa proteins after serotonin stimulation is more transient compared to platelet stimulation with potent agonists like thrombin, collagen and

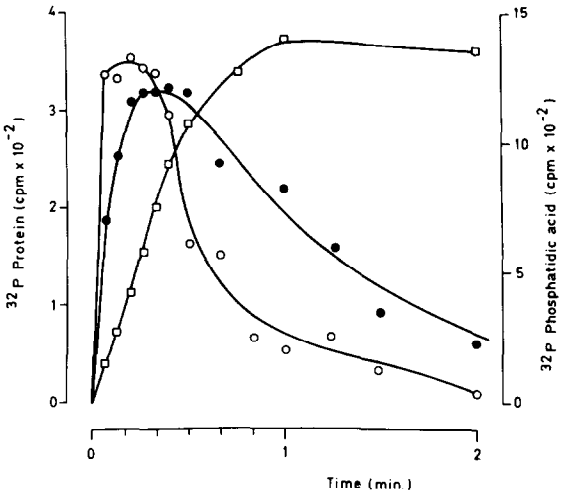

Fig.1. Time course of increase in phosphorylation. Isolated human platelets prelabelled with [^{32}P]orthophosphate were stimulated with 4×10^{-6} M serotonin. (○) ^{32}P-labelled 20 kDa protein, (●) ^{32}P-labelled 40 kDa protein, (□) [^{32}P]phosphatidic acid. Points represent the mean of 2 determinations on the same platelet preparations. The results are representative of 5 separate experiments.

platelet activating factor [5–8,13,14]. The low degree of platelet activation that corresponds with a small activation of the phospholipase C [2,13] could at least in part be responsible for this observation. A similar finding has been reported after stimulation of platelets with arachidonic acid (0.2 μM) another weak agonist [9]. Using this stimulus, at least in this concentration, formation of [^{32}P]PA (~230% above control) and the time course of 40 kDa protein phosphorylation are approximately the same as those found with serotonin (here, [2]).

Pre-incubation of the platelets with subnanomolar concentrations of ketanserin dose-dependently inhibited the serotonin-stimulated ^{32}P-labelling of both the 40 and 20 kDa protein (fig.2). Inhibition curves coincided with the inhibition by ketanserin of serotonin-stimulated ^{32}P-labelling of PA (fig.2). The drug concentration required to inhibit the biochemical processes matched its binding affinity for serotonin-S_2 receptor sites on rat cerebral cortex membranes [15], cat platelet membranes [16] and human platelet membranes [17]. Ketanserin has been described as a

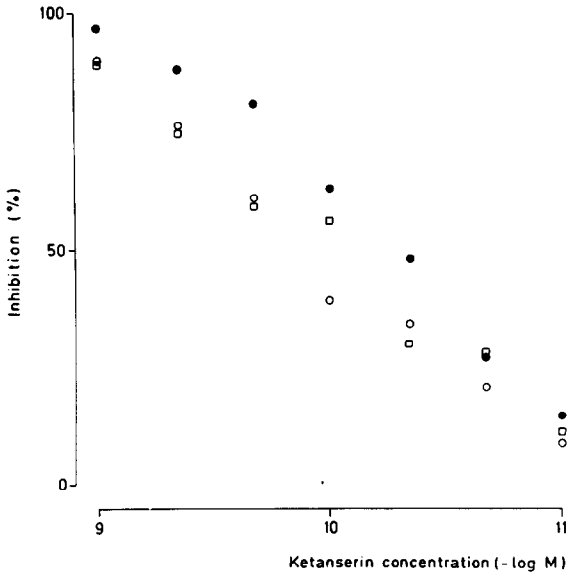

Fig.2. Dose-dependent inhibition by ketanserin of serotonin-induced changes [^{32}P]phosphatidic acid, ^{32}P-labelled 40 kDa and 20 kDa protein. Isolated human platelets labelled with [^{32}P]orthophosphate were pre-incubated for 22 min with the appropriate concentration of ketanserin. Then serotonin (4×10^{-6} M) was added. Samples were taken at 15 s for protein analyses and at 35 s after stimulation for lipid analysis. (○) ^{32}P-labelled 20 kDa protein (mean of 4 determinations), (●) ^{32}P-labelled 40 kDa protein (mean of 4 determinations), (□) [^{32}P]phosphatidic acid (mean of 2 determinations). Results are representative of 3 separate experiments.

new serotonin antagonist which binds primarily to serotonin-S_2 receptor sites; its binding affinity for the latter receptor sites (K_d = 0.4 nM) being at least 25 times higher than its affinity for other neurotransmitter receptor sites [18]. At the sub-nanomolar ketanserin concentration used here no interactions with other receptor sites than the serotonin-S_2 sites are to be expected [18]. The drug was shown in previous studies not to active on non-serotonin dependent human platelet reactions [11], nor to have any direct effect on the phosphorylation systems of the 40 and 20 kDa proteins after stimulation with thrombin (1 unit/ml) or ADP (10^{-5} M) (table 1). Therefore it is concluded that the decrease in the level of ^{32}P-labelled protein after serotonin stimulation in the presence of ketanserin is due to a block of the receptor-agonist coupling.

Table 1

Influence of ketanserin on protein phosphorylation after stimulation of isolated platelets with thrombin and ADP

Addition	40 kDa (cpm)	20 kDa (cpm)
None	275 ± 15	107 ± 2
Thrombin (1 unit/ml)	3050 ± 68	1009 ± 23
Thrombin (1 unit/ml) + ketanserin (10^{-8} M)	3118 ± 59	1003 ± 12
ADP (10^{-5} M)	549 ± 11	300 ± 6
ADP (10^{-5} M) + ketanserin (10^{-8} M)	520 ± 31	315 ± 17

Isolated human platelets prelabelled with [^{32}P]orthophosphate were stimulated with thrombin or ADP for 30 s. The contact time with the drug before stimulation was 22 min. Results represent means ± SD of triplicate samples

Antagonism of the 40 kDa phosphorylation by increasing platelet cAMP levels has been reported [4,5,7–9]. Such an antagonism does not, however, prove the link between the receptor and protein phosphorylation since the latter might be directly regulated by cAMP levels, neither do experiments in which protein phosphorylation could be suppressed by trifluperazine and dibucaine, since in the presence of these drugs the primary biochemical events after receptor occupation (phospholipase C activation and increase in intracellular free Ca^{2+}) took place [5,19]. Evidence in favour of a direct link between receptor occupation and protein phosphorylation was provided by the use of a synthetic DAG and the calcium ionophore A23187. In these experiments it was clearly shown that the products of the early biochemical events after receptor-agonist coupling, DAG and Ca^{2+} could independently enhance phosphorylation of the 40 and 20 kDa protein; a receptor was, however, not involved [20].

In conclusion, stimulation of platelets by serotonin provides us with a model in which receptor occupancy, the primary biochemical events (phospholipase C activation [2] and intracellular free Ca^{2+} increase [21]) and protein phosphorylation are coupled and in which a receptor antagonist can stop the cascade of events comprising the signal transducing system.

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