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DIRECT EVIDENCE FOR THE MODULATION OF HUMAN PLATELET CYTOSOLIC FREE Ca^{2+} BY INTRACELLULAR cyclic AMP PRODUCED WITH A PHOTOACTIVATABLE DERIVATIVE

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SUMMARY We have previously reported that intraplatelet "cyclic AMP jumps" produced with newly synthesized photoactivatable cyclic AMP analogue, inhibited washed rat platelet aggregation and serotonin release as induced by thrombin. Using the same approach on human platelets, thrombin-induced platelet aggregation was dose-dependently inhibited only when a flash was delivered. The mechanism of action of intraplatelet cyclic AMP as resulting from photolysis could be by controlling the level of cytosolic Ca^{2+}. In order to test this hypothesis, the same protocol was used on human platelets preloaded with the internal Ca^{2+} fluorescent indicator, Quin 2, we found that the extent and the rate of the rise of the cytosolic Ca^{2+} induced by thrombin were dramatically decreased, in the presence of the photoactivatable cyclic AMP, only following photoirradiation. In addition, the flashes were produced, in the presence of photoactivatable cyclic AMP, after the thrombin-induced rise of internal Ca^{2+} had reached its peak. In these conditions, photoirradiation caused a rapid fall in fluorescence. These experiments provide the first direct evidence that intracellular cyclic AMP is involved in the control of platelet cytosolic Ca^{2+} by inhibition of its mobilization and by stimulation of its sequestration.

It is now well established that Ca^{2+} is a second messenger involved in the stimulus-induced activation of blood platelets (1). The rapid increase in the cytosolic free Ca^{2+} concentration has been demonstrated by using fluorescent probes or the photoprotein aequorin (2,3). These studies have also shown that the rise in cytosolic Ca^{2+} is largely due to an extracellular Ca^{2+} influx and, to a lesser extent, to a release from the dense tubular system (4-7). These data confirm the results obtained previously (8) or more recently (9), using radio-calcium. On the other hand, cyclic AMP (cAMP) is another important modulator involved in the

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regulation of platelet function (10,11). The most potent inhibitors of the platelet activation processes are prostaglandins I_2, D_2 and E, and forskolin. These compounds are supposed to act through powerful stimulation of platelet adenylate cyclase (12,13). Also dibutyryl cAMP has been proved to inhibit platelet function (14).

Taking advantage of newly synthesized photolabile cyclic nucleotide analogues (15), we have recently presented direct evidence for the inhibitory effect of cAMP on rat platelet aggregation and 5-HT release (16). These derivatives are physiologically inert before irradiation and liberate free cyclic nucleotides upon photolysis. One of the mechanisms by which cAMP regulates platelet functions could be by controlling the level of Ca^{2+} in the cytosol. In this paper, using the same photoactivatable derivative on Quin 2-loaded human platelets, cAMP "concentration jumps" were produced and direct evidence that cAMP affects the platelet cytosolic free Ca^{2+} is presented.

METHODS

Platelet preparation and aggregation : Blood was drawn as elsewhere described (9) from healthy volunteers who had not taken drugs for the previous 2 weeks. Briefly, 3 vol. of the anticoagulant, acid citrate dextrose, were mixed with 7 vol. of blood and centrifuged (200 x g, 10 min, 20°C) to obtain the platelet-rich plasma. The platelets, recovered after centrifugation (800 x g, 18 min, 20°C) of the platelet-rich plasma, were resuspended (3-4 x 10^8/ml) in Ca^{2+}-free Tyrode's buffer containing, in mM : 149 NaCl, 2.6 KCl, 9.5 $NaHCO_3$, glucose 5.5, 0.5 NaH_3PO_4, 0.6 $MgCl_2$ and gelatin (Merck) 2.5 g/l and adjusted to pH 7.4 with 0.25 M HCl. Platelet aggregation experiments were performed (9) with 0.5 ml of the stirred (1100 RPM) platelet suspension. Test compounds in dimethyl sulfoxide (DMSO, final concentration 0.2 %) were first added to the platelet suspension. Single flashes were then delivered as described below (see also 16) after the desired time. Platelets were then stimulated with human thrombin (0.05-0.1 U/ml, Sigma) after the calcium concentration had been increased to 0.3 mM.

Quin 2 loading and fluorescence measurement : The platelet-rich plasma was incubated at 37°C for 20 min in the presence of 7 uM Quin 2 acetoxymethyl ester (Calbiochem). After centrifugation (800 x g, 15 min, 20°C), the platelet pellet was resuspended (0.4-0.8 x 10^8/ml) in Ca^{2+}-free Tyrode's buffer pH 7.4 containing gelatin as above and 10 mM sodium 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid. Fluorescence was measured with 1 ml of the suspension maintained at 37°C in a model SLM Aminco SPF 500C spectrofluometer in the ratio mode. Fluorescence excitation was at 339 nm and emission was measured every 2 seconds at 492 nm. The data were printed on a Hewlett-Packard model 7470 A plotter. The gaps in the trace indicate periods for light irradiation. The calibration of intracellular fluorescence as a function of calcium ([Ca]i) was

performed essentially as described previously (17) using the following formula :

$$[Ca]i = 115\ (F-F_{min})/(F_{max}-F)$$

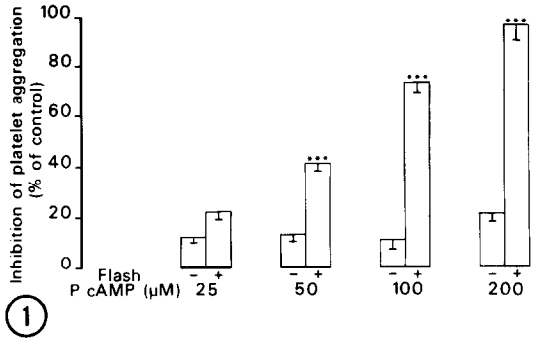
where 115 nM is the K_d of the Ca-Quin 2 complex. F is the fluorescence of the intact cell suspension. F_{max} was obtained following addition of 70 nM of ionomycin (Calbiochem) and F_{min} after addition of 0.5 mM $MnCl_2$ (18).

Intraplatelet jumps of cAMP concentration : 4,5-dimethoxy-2-nitrobenzyl ester of cyclic AMP (P.cAMP, a generous gift of Dr J.M. Nerbonne, St Louis, MO, presently available from Molecular Probes, OR) liberates free cAMP upon irradiation. The photoactivatable derivative was dissolved in DMSO and added to the platelet suspension (final DMSO concentration : 0.2 %) at the indicated concentration (25-200 uM). The cuvettes containing the suspension were magnetically stirred and the flashes were produced through a 300 nM cut-off filter (Schott WG 295) as previously described (15,16) using a xenon short-arc flash lamp ; the flash duration was 1 msec ; total energy 1 J. For aggregation experiments, the light source was disposed, as previously described (16), above the cuvette in the aggregometer. For internal Ca^{2+} monitoring, it took only 3-6 sec to irradiate the cuvette and to replace it in the fluorometer. On a few occasions, Quin 2-loaded platelets were monitored for their aggregation pattern as described above and for their serotonin content or release using our voltametric procedure described previously (19).

RESULTS

Washed human platelets were submitted to several flashes through the 300 nm cut-off filter, in the presence of 1 mM external Ca^{2+} and 0.2 % DMSO (the vehicle of P.cAMP). It was thus verified, as already described for rat platelets (16), that the filter was necessary to preserve the platelet integrity as revealed by the normal aggregation and endogenous 5-HT release patterns obtained upon thrombin stimulation.

In the experiments of Fig.1, washed human platelets were incubated with varying concentrations of P.cAMP for 2.5 min before thrombin addition. When needed a single flash was delivered 30 sec before thrombin addition. The results are expressed as percent inhibition compared to control conditions. It is clear that inhibition of aggregation was obtained only when a flash was delivered. The analogue itself was without effect. Similarly, P.cAMP previously irradiated and then added to the platelet suspension had no effect. These results suggest that cAMP liberated by the flash has an inhibitory effect on human platelets as was the case for washed rat platelets (16).



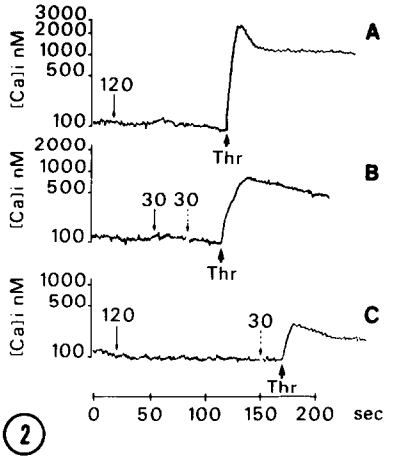


Figure 1 . Thrombin-induced platelet aggregation in the presence of different concentrations of the photoactivatable cyclic AMP (P.cAMP) with or without flash. P.cAMP was added in dimethylsulfoxide (<0.2 %) to washed human platelets resuspended (3-4 x 10^8/ml) in Ca^{2+}-free Tyrode's buffer pH 7.4 containing gelatin (see Methods). After 2.5 min incubation, a single flash was or was not delivered and human thrombin (0.05-0.1 U/ml) was added in the presence of 0.3 mM external Ca^{2+}. The data were obtained from the results of 3 experiments carried out in duplicate with platelets from different donnors and are expressed as percent inhibition (mean ± SD) with respect to control. ***P<0.005 with unpaired Student's t-test.

Figure 2 . Effect of the time before photoirradiation on the variation of the internal Ca^{2+} concentration of Quin 2-loaded human platelets in the presence of P.cAMP. Fluorescence was measured and calibrated as explained in Methods. 100 uM P.cAMP were added (thin arrow) to Quin 2-loaded platelets (0.4-0.8 x 10^8/ml) after temperature equilibration (37°C) in the presence of 1 mM Ca^{2+}. In A, no flash has been delivered before platelet stimulation by 0.2-0.7 U/ml human thrombin (Thr, thick arrow) and 120 sec after addition of the photoactivatable derivative. In B, 30 sec after P.cAMP addition, a single flash (dashed arrow) was delivered and 30 sec later thrombin was added. In C, the preincubation with the P.cAMP was realized 120 sec before light irradiation and thrombin addition. The numbers indicate the time elapsed after addition of P.cAMP or photoirradiation. In B and C the time in the presence of P.cAMP varied from 30 to 120 sec while the time after flashing remained constant (30 sec). The gaps in the traces indicate periods when the cuvettes were handled and irradiated.

Using Quin 2-loaded human platelets, it was also verified that after several flashes, the classical rise of fluorescence was effectively recorded following the addition of 0.2-0.7 U/ml thrombin in the presence of 1 mM external Ca^{2+}. These results indicate that Quin 2 was not destroyed by the flash light and thus, the probe had kept its properties. In the experiments ilustrated in Fig.2, it can be seen that Quin 2-loaded platelets, incubated in the presence of P.cAMP, presented a normal rise in their cytosolic Ca^{2+} ([Ca]i) a few seconds after thrombin stimulation if

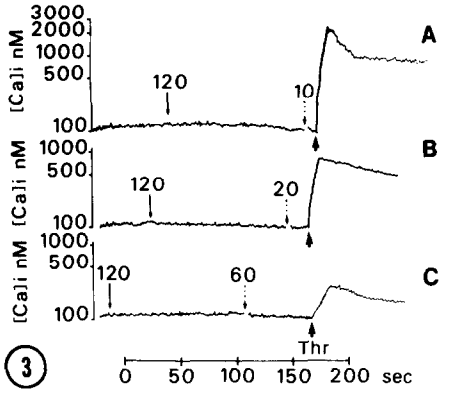
no flash had been delivered. The [Ca]i peak level was estimated to be in the range 1.5-3 uM. Thus, it is clear that platelet-evoked increase in cytoplasmic Ca^{2+} is inhibited only when a flash is delivered. When Quin 2-loaded platelets were incubated with 200 uM P.cAMP which had been previously extensively irradiated with U.V. light (5 min), no significant inhibition of the rise of the fluorescent signal could be observed. These results indicate that, as for aggregation, extracellularly cAMP concentration increased, as could result from the photolysis, had no effect on the platelet-evoked rise in cytosolic free Ca^{2+}.

Figure 2 also shows that the incubation time in the presence of P.cAMP before the flash was of importance. Indeed, when this time period was as long as 2 min the inhibition of the thrombin-induced rise of fluorescence was far greater than for a shorter preincubation time (30 sec). In these experiments the time after the flash prior to thrombin addition remained constant (30 sec) as did the P.cAMP concentration (100 uM).

Also of great importance is the time after the flash as suggested by the results depicted in Fig.3. In these experiments, the platelets were incubated with 100 uM P.cAMP, a single flash was then delivered and thrombin was added after a varying time. In Fig.3, only the experiments corresponding to the 10, 20 and 60 sec times are illustrated. The rate as well as the magnitude of the increase in cytoplasmic Ca^{2+} were inhibited.

Using the same technique we have reported that, when a flash was delivered after the beginning of thrombin-induced aggregation performed in the presence of P.cAMP, a reversal of the aggregation process occurred. In order to see whether internal Ca^{2+} could play a role in this observation, we repeated these experiments with Quin 2-loaded human platelets.

In Fig.4, platelets loaded with Quin 2 were stimulated with thrombin in the presence of 200 uM P.cAMP. Although the increase of cytosolic Ca^{2+} had reached its plateau, when the flash was delivered, the fluorescence rapidly decreased. The same observation was made when irradiation occurred



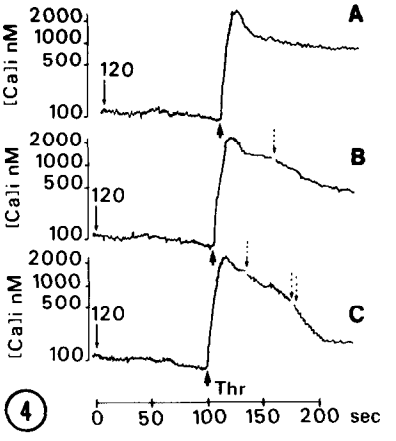


Figure 3 . Effect of the time after photoirradiation on the variation of the interval Ca^{2+} of Quin 2-loaded human platelets in the presence of P.cAMP. Same protocol as for Fig.2 except that the incubation time with 100 uM P.cAMP was maintained constant (120 sec) and the time after flashing ranged from 10 sec (A), 20 sec (B) to 60 sec (C).

Figure 4 . Effect of cAMP jumps produced after platelet stimulation. Quin 2-loaded human platelets were incubated for 120 sec with 100 uM P.cAMP (thin arrow). Platelets were stimulated (thick arrow) with human thrombin (0.2-0.7 U/ml). (A) shows a typical trace without flash. In (B), 50 sec after the thrombin-induced rise of [Cali, a single flash was delivered and it was followed by a decrease in fluorescence. In (C), a single flash was delivered at 30 sec which induced as in (B) a decrease of the [Cali, and 40 sec later, 2 flashes caused an additional faster fall in [Cali.

at different times. With two flashes, the rate in the decrease of the intracellular Ca^{2+} was even faster. Although the internal Ca^{2+} level tended to slightly decrease in control experiments without flash, in every case when flashes were delivered, they caused a rapid fall of the cytoplasmic Ca^{2+}.

DISCUSSION

We report in this paper that the production of intracellular cAMP dramatically inhibits the thrombin-induced rise of the platelet cytoplasmic free Ca^{2+}. The latter has been monitored in human platelets by means of the fluorescent dye Quin 2 following the works of Rink et al (2, see also 4,5). As developed by Tsien (17), Quin 2 acetoxymethyl ester enters cells where it is hydrolyzed by intracellular esterase. The fluorescence of the resulting Ca^{2+} chelator Quin 2, trapped in the

cytoplasm, is selectively enhanced in the presence of Ca^{2+}. On the other hand, to generate cAMP production in platelets, we used the 4,5-dimethoxy-2-nitrobenzyl ester of cyclic AMP (P.cAMP) which, upon photolysis, liberates free cyclic AMP. These newly synthesized photolabile cyclic nucleotide analogues have been recently described (15). These derivatives are almost physiologically inert before irradiation and liberate free cyclic nucleotides on absorption of a photon. Using such an experimental approach, we have previously reported that intracellular cAMP, resulting from photolysis, dramatically inhibited platelet aggregation and serotonin release of washed rat platelets (16). These inhibitory effects could probably be ascribed to cAMP and could not be attributed to the by-products of the photoreaction (H^+, 4,5-dimethoxy-2-nitrosobenzaldehyde) (15,16).

We consistently found that the platelet behavior was slightly inhibited (10-20 %) in the presence of Quin 2 and P.cAMP. We have reported in an earlier paper this fact concerning aggregation and release of washed rat platelets for high concentrations of P.cAMP (16). It is not known whether the inhibitory effect caused by P.cAMP itself is non-specific or whether it is related to the obvious structural similarities of the analogue and free cAMP. As for Quin 2, the inhibitory effects ascribable to the Ca^{2+} buffering properties of the molecule have been discussed (6,20,21). It was for these reasons that, in our conditions, low concentrations of both P.cAMP (50-100 uM) and Quin 2 (7 uM) were utilized as well as slightly higher concentrations of thrombin (0.2-0.7 U/ml instead of 0.01-0.1 U/ml). Concerning the thrombin-induced aggregation of Quin 2-loaded human platelets, our results confirm previous data obtained using normal rat platelets (16) : we found that intracellular cAMP, resulting from photolysis, inhibits aggregation. These results concord well with earlier reported observations obtained with human platelets using dibutyryl cAMP (14). With respect to the cytoplasmic Ca^{2+}, in studies on the effects of the incubation time, the results suggest that

the incubation time before the flash determines the intraplatelet concentration of the P.cAMP. These data suggest that, following a 120 sec incubation before flashing, a sufficient amount of the photoactivatable derivative has entered the platelets. On the other hand, the incubation time after irradiation represents the time during which the intraplatelet effectors are exposed to free cAMP liberated by photolysis. This was also confirmed by the control experiments where the previously extensively irradiated P.cAMP had no effect on the cytoplasmic Ca^{2+}. These latter observations are consistent with the well fact that free cAMP does not easily permeate through cell membranes. It was for these reasons that more hydrophobic stable derivatives, such as dibutyryl cAMP, were used (1,14). By contrast, using this photoactivatable cAMP derivative at concentrations of 50-100 uM, we have shown that a single flash results in a drastic inhibition of the rise of cytoplasmic free Ca^{2+}. In addition, there was no need of phosphodiesterase inhibitors to reinforce the effects.

We have made no direct measurements of cAMP actually produced intracellularly following a single flash. Because the reaction efficiency is relatively insensitive to environment, it is likely that the intracellular concentration jumps are at least as large as those produced extracellularly. Consequently, taking into account that approx. 5 % (15) of the molecules are converted per flash, intraplatelet concentration steps in the range of 2-10 uM could be assumed.

Our results demonstrate a direct link between cAMP and Ca^{2+} mobilization in intact platelets. This had already been strongly suggested by means of stimulation of platelet adenylate cyclase activity with PGD_2, E_1, forskolin or dibutyryl cAMP (10,11,22). These agents cause a drastic reduction of platelet cytoplasmic Ca^{2+}. Further work is necessary to determine how cAMP inhibits the rise in cytoplasmic Ca^{2+}. However, our results confirm previous studies indicating that cAMP may inhibit either the intracellular release of Ca^{2+} or its influx from the medium or both (23-26). The reversal of thrombin-induced myosin phosphorylation and the

assembly of cytoskeletal structures in platelets by stimulation of adenylate cyclase activity indicated that the mechanism of action of cAMP might be to rapidly remove from the cytoplasm the Ca^{2+} which had been released by thrombin. This is illustrated in Fig.4 where the platelet cytoplasmic Ca^{2+} tended to reach back its basal level when flashes were delivered after thrombin addition. These results confirm previous data reporting that adenylate cyclase stimulants (26,27) enhance the Ca^{2+} sequestration. This also concords with the deaggregating properties of these agents (22) and intracellular cAMP produced on previously aggregated platelets (16). By analogy with the sarcoplasmic reticulum-T-tubule system of muscle, it is assumed that the platelet dense tubular system could play this role since it contained a Ca^{2+} transport mechanism (23,28,29). Ca^{2+} uptake by platelet membrane microsome vesicles has been reported to be enhanced in the presence of the catalytic subunit of the cAMP dependent kinase (30). In these conditions, there is a phosphorylation of a 23 KD protein of these platelet membranes. Recently, Enouf et al (31) have provided strong evidence for a Ca ATPase activity from a particular membrane fraction which could originate from the surface-connected canalicular system (32,33). Holmsen recently reported, using porcine platelets, that mitochondria, which might be good candidates in the resequestration of the cytoplasmic Ca^{2+}, may be of no importance in this process (34).

In conclusion, it is of interest to note that the photoactivatable cAMP derivative enabled a direct effect of cAMP on cytosolic $Ca^{2+}$to be demonstrated. Further work is needed to determine how cAMP acts on cAMP-dependent enzymes and structures to generate its regulatory control.

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