Volume 219, number 2, 283–288 FEB 04912 July 1987

The synergistic effect of serotonin and epinephrine on the human platelet at the level of signal transduction

D. de Chaffoy de Courcelles, P. Roevens, H. Van Belle and F. De Clerck

Department of Biochemistry, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

Received 24 March 1987

Simultaneous addition to platelets of submaximal amounts of excitatory agonists acts synergistically in provoking secretory and aggregatory responses. By measuring changes in intracellular free Ca^{2+} concentration, inositol phospholipid metabolism and protein phosphorylation, we verified whether synergism could be evidenced at the level of signal transduction. Challenging platelets with epinephrine only induced minor changes on the measured parameters. However, when added together with serotonin, epinephrine amplified mobilisation of intracellular Ca^{2+}, PA formation, PIP formation, protein kinase C and myosin light chain kinase activity as compared to the alterations induced by serotonin alone. It is concluded that synergistic effects on simultaneous addition of serotonin and epinephrine might originate at the level of signal transduction.

Platelet; Serotonin; Epinephrine; Inositol phospholipid; Ketanserin; (Human)

1. INTRODUCTION

Synergistic interactions between excitatory agonists on platelet aggregation and secretion are widely reported [1–6]. The physiological importance of this phenomenon is apparent since under conditions of haemostatic stress, minor amounts of platelet agonists (as such ineffective) might by synergism lead to aggregate formation. The site at which separate agonists act synergistically in the pathway leading to aggregation after receptor activation is hitherto unknown. In a recent report [7] evidence was presented for the synergistic effect of different excitatory agonists in increasing intracellular free Ca^{2+} concentration. Synergistic actions at the level of signal transduction might be important especially since a causal relationship between receptor-induced breakdown of inositol phospholipids and the increase in intracellular Ca^{2+} is hypothesized. Indeed, inositol triphosphate, a phosphodiesteratic breakdown product of phosphatidylinositol 4,5-bisphosphate induces Ca^{2+} release from platelet intracellular Ca^{2+} stores [8–11]. Beside the inositol phosphates, diacylglycerol is formed. This neutral lipid activates protein kinase C [12,13] and is rapidly phosphorylated to PA. PA and lyso-PA also cause release of Ca^{2+} from intracellular stores [14].

Most platelet excitatory agonists have phosphodiesteratic breakdown of inositol phospholipids involved in signal transduction [15]. For epinephrine, formation of inositol phosphates was demonstrated and the diacylglycerol formed activated protein kinase C but only in the presence of fibrinogen [16–18] and as a consequence of activation of a chain of events leading to stimulation of phospholipase A_2 and generation of excitatory

Correspondence address: D. de Chaffoy de Courcelles, Dept of Biochemistry, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PA, phosphatic acid

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

prostaglandin endoperoxides and thromboxane A_2 [17,18]. The adrenergic receptor of the platelet closely resembles the α_2-receptor [19,20]. Negative coupling to adenylate cyclase was demonstrated [21], and its role in platelet activation was questioned [7,22].

As shown in earlier work, platelet activation by serotonin involved the breakdown of inositol phospholipids as a signal transduction system [23–25].

We now report that the synergistic effect of serotonin and epinephrine found on platelet secretion and aggregation [2,3,6] might originate at the level of signal transduction.

2. EXPERIMENTAL

Human venous blood and platelet rich plasma (PRP) was obtained as described in [24].

For intracellular Ca^{2+} measurements, PRP was incubated with 15 μM Quin-2 acetoxymethyl ester at 37°C for 12 min. Platelets were separated from plasma and extracellular dye by gel filtration on a Sepharose-2B column (2.5 × 18 cm) equilibrated with 125 mM NaCl, 2.7 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 25 mM Hepes, 10 mM glucose, 0.1 mM EGTA, 0.1% BSA at pH 7.4. The platelet concentration was adjusted to 2.2×10^8/ml and 1.2 μmol $CaCl_2$/ml was added. Platelets were incubated for 45–60 min at 37°C before starting measurements. Fluorescence readings were recorded at 37°C using a thermostatted cuvette in a purpose-modified Technicon fluoronephelometer (excitation at 340 nm; emission at 490 nm). The measurements were corrected for dye leakage using 100 μM Mn^{2+} and 200 μM CaDTPA [26].

Platelets for labelling experiments were prepared by gel filtration as described above and incubated for the times indicated with approx. 500 μCi [^{32}P]orthophosphate/ml. Phospholipid extraction, phospholipid analysis and phosphoprotein analysis were performed exactly as described in [24,25].

Platelet aggregation, release of intraplatelet ATP and biosynthesis of thromboxane A_2 in human PRP were assessed as described [6,27]. Aggregation was assessed turbidometrically and quantified by measuring the slope ($\Delta\%T$/min) and the maximum ($\Delta\%T$) of the first and the second wave of aggregation. The amount of TXB_2 in the cell-free plasma was measured by radioimmunoassay [27]. For the simultaneous measurement of aggregation and ATP release, luciferin-luciferase (4 mg/ml) was incorporated in the PRP sample before the induction of aggregation. The total amount of ATP released was calculated from the luminescence and is expressed as μM ATP/1.4×10^8 platelets.

Quin-2 acetoxymethyl ester was obtained from Calbiochem (La Jolla, USA). Serotonin was from Janssen Chimica (Beerse, Belgium). Epinephrine came from Sigma and luciferin-luciferase from Chronolog (Havertown, USA). Ketanserin was obtained from Janssen Pharmaceutica (Beerse, Belgium).

3. RESULTS

Fig.1 illustrates the synergistic effect of epinephrine and serotonin on platelet primary aggregation, release reaction and secondary release-dependent platelet recruitment. Serotonin (5×10^{-6} M) and epinephrine (1.5×10^{-7} M) at concentrations which induced respectively shape change followed by a reversible aggregation and a weak primary aggregation response, produced a strong biphasic aggregation/release reaction when combined; both the first wave of the response and the release-dependent secondary wave of aggregation were amplified. Although the platelet response to a large concentration range of epinephrine was amplified by serotonin (not shown), the synergism was most apparent using low concentrations of epinephrine (fig.1).

As shown in [7] for combinations of epinephrine with U46619, thrombin, ADP or vasopressin, we verified whether the increase in intracellular Ca^{2+} on addition of serotonin to human platelets was amplified by epinephrine. Fig.2 illustrates that serotonin when added alone increased the fluorescence response. The addition of epinephrine (10^{-5} M) had no effect on the Quin-2 signal, when added together with serotonin (10^{-6} M) the response was increased. The serotonin-S_2 receptor antagonist, ketanserin (3×10^{-8} M), completely inhibited the synergistic effect of both agonists on Ca^{2+} mobilisation (fig.2).

In earlier reports we found that on addition of serotonin to human platelets prelabelled with [^{32}P]orthophosphate for 70 min, there was a rapid

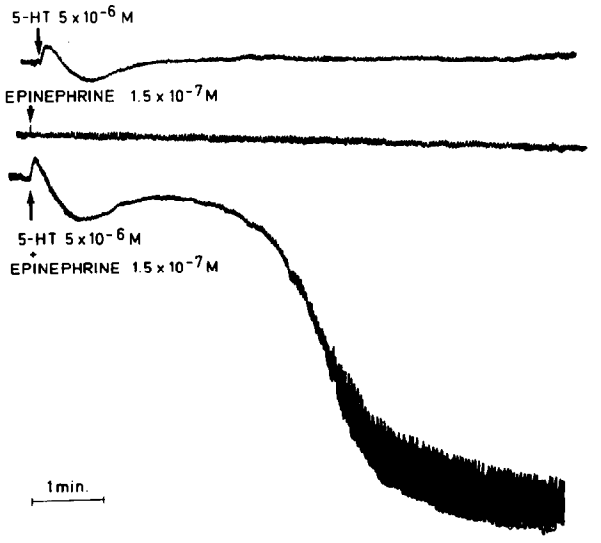

Fig.1. Representative tracings obtained on the same human PRP at paired time intervals. Shape change followed by reversible aggregation induced by serotonin (5-HT, 5×10^{-6} M); weak, primary aggregation induced by epinephrine (EPI, 1.5×10^{-7} M). Amplification of the first wave and induction of release-associated irreversible aggregation by combined 5-HT + epinephrine. First wave of aggregation: 5-HT, 14.8 ± 2.5%T/min; EPI, 1.5 ±0.4%T/min; 5-HT + EPI, 29.8 ± 2.5%T/min. ATP release (at 3 min): 5-HT, 0 ± 0; EPI, 0 ± 0; 5-HT + EPI, $1.57 \pm 0.9 \times 10^{-6}$ M (mean ± SE, $n = 4$; $p < 0.05$ vs single agonists). Plasma TXB_2 (at 3–5 min in pg/100 μl): 5-HT, 59.6 ± 7.2; EPI, 47.7 ± 3.4; 5-HT + EPI, 4400 ± 605 (mean ± SE, $n = 4$; $p <$ 0.05 vs single agonists).

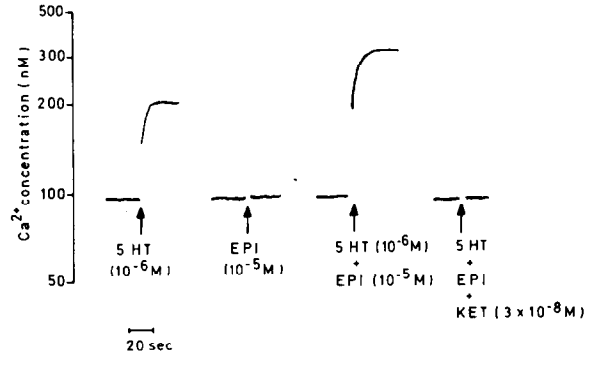

Fig.2. Platelets loaded with Quin-2 were stimulated with either (A) serotonin (5-HT), (B) epinephrine (EPI), (C) a mixture of 5-HT and EPI or (D) a mixture of 5-HT and EPI after preincubation for 2 min with ketanserin (KTZ).

increase in [^{32}P]PA reflecting formation of diacylglycerol [23,24]. Fig.3 and table 1 illustrate that in similar experimental conditions, epinephrine only slightly increased [^{32}P]PA formation compared to the increase when platelets were challenged with serotonin alone. When added simultaneously [^{32}P]PA formation was amplified. The stimulatory effect of both agonists was completely abolished by ketanserin. The amplificatory effect of epinephrine on Ca^{2+} mobilisation and PA formation was maximal at 3×10^{-6} M (not shown).

Serotonin also induces an increase in protein kinase C and myosin light chain kinase activity that in ^{32}P-labelled platelets are reflected by an increased phosphorylation of the 47 kDa and 20 kDa proteins [23]. Stimulation of platelets with serotonin further increases their PIP and decreases their PIP_2 content as reflected by changes in ^{32}P labelling [25]. Table 1 illustrates that protein phosphorylation and [^{32}P]PIP formation were amplified while [^{32}P]PIP_2 no longer decreased but increased. Epinephrine had a small but significant effect on PA formation and protein phosphorylation. The ^{32}P content of phosphatidylinositol (PI) and phosphatidylcholine was not affected by serotonin [25], epinephrine or simultaneous addition of both agonists (not shown).

Prelabelling platelets with [^{32}P]orthophosphate for only 5 min, enables quantification of

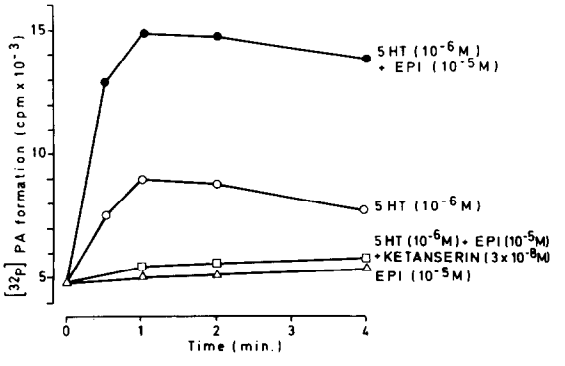

Fig.3. Platelets prelabelled with [^{32}P]orthophosphate for 70 min were stimulated with either serotonin (5-HT), epinephrine (EPI), a mixture of 5-HT and EPI or a mixture of 5-HT and EPI after preincubation for 10 min with ketanserin. At the times indicated duplicate samples were taken. Results are representative for 5 separate experiments.

Table 1

The amplificatory effect of serotonin and epinephrine on inositol phospholipid metabolism, protein phosphorylation and Ca^{2+} mobilisation

	^{32}P incorporation (% of control)			
	Epi (3×10^{-6} M)	5-HT (10^{-6} M)		Epi (3×10^{-6} M) + 5-HT (10^{-6} M)
PA	116 ± 4	223 ± 22	$p < 0.005$	324 ± 36
PIP	102 ± 1 (n.s.)	111 ± 3	$p < 0.02$	120 ± 5
PIP_2	101 ± 1 (n.s.)	93 ± 1	$p < 0.005$	107 ± 2
40 kDa proteins	111 ± 4	165 ± 20	$p < 0.01$	295 ± 46
20 kDa proteins	119 ± 8	156 ± 11	$p < 0.005$	219 ± 18
Ca^{2+}	no increase	197 ± 28	$p < 0.01$	303 ± 52
	(Increase in intracellular Ca^{2+}, nM)			

For ^{32}P-incorporation studies, platelets were prelabelled for 70 min. Platelets were challenged either with epinephrine (Epi), serotonin (5-HT), a mix of Epi and 5-HT or solvent (9% NaCl). After 50 s stimulation a single sample was taken for protein analysis; after 55 s duplicate samples were taken for lipid analysis. For data calculation, the duplicate values from lipid analysis were averaged. The ^{32}P incorporation on addition of 9% NaCl was set at 100% (control) for each experiment and the corresponding values after addition of agonists were calculated. Values represent mean ± SE of 4 experiments. Intracellular Ca^{2+} concentrations were obtained as described in section 2. Agonist-induced increase was measured 20 s after its addition. The intracellular free Ca^{2+} concentration in the unstimulated platelets was 97 ± 7 nM. Results represent means ± SE ($n = 6$) of agonist-induced increase in intracellular free Ca^{2+}. Except for those indicated by n.s., all the values differed significantly ($p < 0.05$ in paired Student's t-test) from controls. The indicated p values were calculated by pairing the data obtained after addition of 5-HT and Epi + 5-HT

serotonin-induced PI turnover [25]. Fig.4 illustrates that besides the amplificatory effect on PA formation, the simultaneous addition of epinephrine increased the serotonin-induced synthesis process of PI.

4. DISCUSSION

As reported earlier [2,3,6], serotonin and epinephrine act synergistically on platelet aggregation and release reaction. The use of Quin-2 as a probe for measuring the intracellular free Ca^{2+} concentration did not allow us to measure increases in Ca^{2+} on stimulation of isolated human platelets with epinephrine. These findings are in accordance with others [7,28–30]. In view of reported epinephrine-induced changes in Ca^{2+} using other probes [29,30], a local small increase in intracellular Ca^{2+} might occur. The amplificatory effect of epinephrine on serotonin-induced Ca^{2+} mobilisation was however detectable by Quin-2 fluorescence as is the case for all excitatory platelet agonists that involve inositol phospholipid breakdown and Ca^{2+} mobilisation in their signal transducing system.

On stimulation of ^{32}P-labelled platelets with epinephrine, increases in [^{32}P]PA formation and 47 kDa protein phosphorylation as measures for phospholipase C and protein kinase C activity were very low (figs 3,4 and table 1) as compared to those

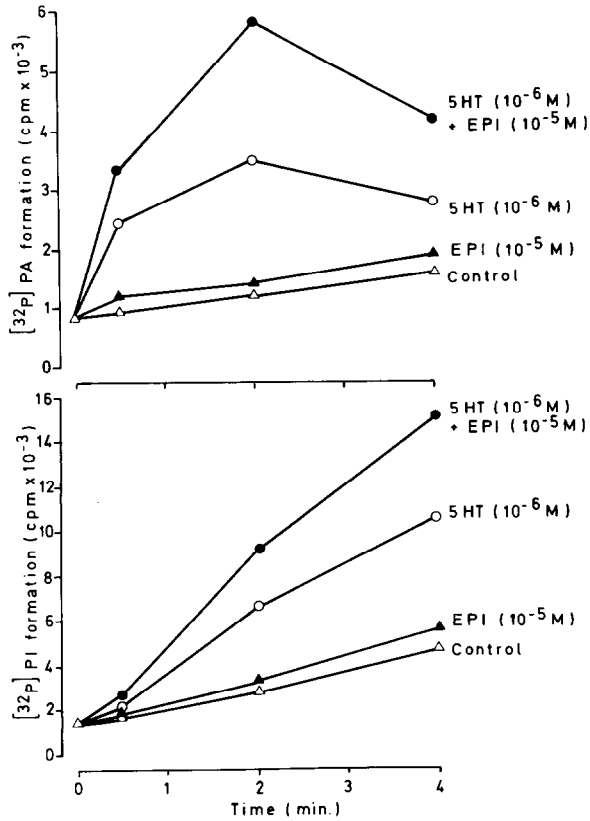

Fig.4. Platelets were preincubated with [^{32}P]orthophosphate for 5 min. Serotonin (5-HT), epinephrine (EPI), a mixture of EPI and 5-HT or the solvent (9‰ NaCl, control) were added (at 0 time on scale). At the times indicated duplicate samples were taken. Results are representative for 3 separate experiments.

found on addition of other strong platelet agonists [12,31–33]. An amplificatory effect of epinephrine on serotonin-induced PA and PIP formation as on 47 kDa and 20 kDa protein phosphorylation was evident (figs 3,4 and table 1). Remarkably, the serotonin-induced PIP_2 breakdown was not amplified by epinephrine (table 1): simultaneous addition of both agonists increased PIP_2 formation. In view of the increased PIP formation when agonists are added together compared to serotonin alone, it is tempting to speculate that the amplificatory effect ascribes to an increase in inositol phospholipid kinase activity thereby elevating the substrate availability for phospholipase C.

As an alternative the involvement of guanine nucleotide-binding proteins should be hypothesized since Ni, the inhibitory guanine nucleotide-binding protein for adenylate cyclase, has been shown to be involved in inositol phospholipid breakdown [34–36]. Since epinephrine itself is not able to decrease cAMP [21] and inhibition of adenylate cyclase per se cannot substitute for epinephrine in the synergistic effect of combined agonists on intracellular Ca^{2+} increase [7], a modulatory effect at the level of cAMP on amplification is rather unlikely.

In conclusion we found that the excitatory platelet signal transduction induced by activation of the serotonin-S_2 receptor is amplified by epinephrine. Epinephrine itself had minor effects on this signal transducing system. The serotonin-S_2 receptor antagonist ketanserin abolished the synergistic response of serotonin and epinephrine.

ACKNOWLEDGEMENTS

Mrs A. Nuyts and L. Geentjens are acknowledged for the careful preparation of this manuscript.

REFERENCES

[1] Niewiarowski, S. and Thomas, D.P. (1966) Nature 212, 1544–1547.
[2] Baumgartner, H.R. and Born, G.V.R. (1968) Nature 218, 137–141.
[3] Packham, M.A., Guccione, M.A., Chang, P.Y. and Mustard, J.F. (1973) Am. J. Physiol. 225, 38–47.
[4] Grant, J.A. and Scrutton, M.C. (1980) Br. J. Haematol. 44, 109–125.
[5] Huang, E.M. and Detwiler, T.C. (1981) Blood 57, 685–691.
[6] De Clerck, F., David, J.L. and Janssen, P.A.J. (1982) Agents Actions 12, 388–397.
[7] Thompson, N.T., Scrutton, M.C. and Wallis, R.B. (1986) Eur. J. Biochem. 161, 399–408.
[8] Brass, L. and Joseph, S.K. (1985) J. Biol. Chem. 260, 15172–15179.
[9] O'Rourke, F., Halenda, S., Zavoico, G. and Feinstein, M. (1985) J. Biol. Chem. 260, 956–962.
[10] Adunyah, S.E. and Dean, W.L. (1985) Biochem. Biophys. Res. Commun. 128, 1274–1280.
[11] Authi, K.S. and Crawford, N. (1985) Biochem. J. 230, 247–253.
[12] Sano, K., Takai, Y., Yamanishi, J. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010–2013.

[13] Nishizuka, Y. (1984) Nature 308, 693–698.
[14] Brass, L.F. and Laposata, M. (1987) Biochem. Biophys. Res. Commun. 142, 7–14.
[15] Siess, W. (1986) Biochem. Pharmacol. 35, 3184–3187.
[16] Siess, W. and Weber, P.C. (1984) J. Biol. Chem. 259, 8286–8292.
[17] Sweatt, J.D., Blair, I.A., Cragoe, E.J. and Limbird, L.E. (1986) J. Biol. Chem. 261, 8660–8666.
[18] Banga, H.S., Simons, E.R., Brass, L.E. and Rittenhouse, S.E. (1986) Proc. Natl. Acad. Sci. USA 83, 9197–9201.
[19] Clare, K.A., Scrutton, M.C. and Thompson, N.T. (1984) Br. J. Pharmacol. 82, 467–476.
[20] Hamilton, C.A. and Reid, J.L. (1986) Br. J. Clin. Pharmacol. 22, 623–626.
[21] Haslam, R.J. (1975) Ciba Found. Symp. 35, 121–143.
[22] Motulsky, H.J., Shattil, S.J., Ferry, N., Rozansky, D. and Insel, P.A. (1986) Mol. Pharmacol. 29, 1–6.
[23] De Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1984) FEBS Lett. 171, 289–292.
[24] De Chaffoy de Courcelles, D., Leysen, J.E., De Clerck, F., Van Belle, H. and Janssen, P.A.J. (1985) J. Biol. Chem. 260, 7603–7608.
[25] De Chaffoy de Courcelles, D., Roevens, P., Wynants, J. and Van Belle, H. (1987) Biochim. Biophys. Acta 927, 291–302.
[26] Rink, T.J. and Pozzan, T. (1985) Cell Calcium 6, 133–144.
[27] De Clerck, F., Xhonneux, B., Leysen, J. and Janssen, P.A.J. (1984) Biochem. Pharmacol. 33, 2807–2811.
[28] Brydon, L.J., Pollock, W.K., Shaw, A.M., Drummond, A.H. and MacIntyre, D.E. (1983) Thromb. Haemostas. 50, 276.
[29] Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W. (1986) J. Clin. Invest. 77, 878–886.
[30] Sweatt, J.D., Connolly, T.M., Cragoe, E.J. and Limbird, L.E. (1986) J. Biol. Chem. 261, 8667–8673.
[31] Siess, W., Siegel, F.L. and Lapetina, E.G. (1983) J. Biol. Chem. 258, 11236–11242.
[32] Lapetina, E.G. and Siegel, F.L. (1983) J. Biol. Chem. 258, 7241–7244.
[33] Siess, W., Boehlig, B., Weber, P.C. and Lapetina, E.G. (1985) Blood 65, 1141–1148.
[34] Lapetina, E.G., Reep, B. and Chang, K.-J. (1986) Proc. Natl. Acad. Sci. USA 83, 5880–5883.
[35] Lapetina, E.G. (1986) Biochim. Biophys. Acta 884, 219–224.
[36] Brass, L.F., Laposata, M., Banga, H.S. and Rittenhouse, S.E. (1986) J. Biol. Chem. 261, 16838–16847.