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THE SYNERGISM BETWEEN PROTEIN KINASE C ACTIVATION AND CALCIUM MOBILIZATION IN PLATELET ACTIVATION.

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It has been shown that Ca^{2+}mobilization and protein kinase C (C kinase) activation act synergistically to elicit the full physiological response in several systems. That was first demonstrated for the release of serotonin from platelets. To clarify whether or not such synergism can be extended to aggregation and contrast platelet functions with cytosolic free Ca^{2+}levels($[Ca^{2+}]i$), we observed platelet aggregation,serotonin release and $[Ca^{2+}]i$ simultaneously by using platelets loaded with ^{14}C-serotonin and Ca^{2+}-sensitive photoprotein aequorin. To induce C kinase activation and Ca^{2+} mobilization independently,we used the exogenous addition of 12-O-tetradecanoylphorbol 13-acetate(TPA) for the former and a Ca^{2+}ionophore,ionomycin,for the latter. Platelet-rich plasma was obtained from healthy human blood and was preincuvated with 1μM ^{14}C-serotonin for 45min. Then aequorin was introduced into platelets by incubation with 10mM EGTA and 5mM ATP as described by Johnson et al. Finally,gel-filtered platelets(GFP) were prepared and the count was adjusted to $3x10^8$/ml with Hepes-Tyrode's buffer containing 1mM Ca^{2+}. GFP were preincubated with aspirin (100μg/ml) for 5 minutes,and purified human fibrinogen(500μg/ml) was added shortly before agonists. Aggregation and aequorin signal were measured by using Chronolog P.I.C.A. The synergistic effects of TPA(50-200nM) and ionomycin(50-200nM) were evident both in aggregation and serotonin release. GFP stimulated by 50nM TPA or 50nM ionomycin alone did not aggregate and released little serotonin. But when they were added together,marked aggregation and release reaction were observed. Aggregation was partially affected by apyrase(2mg/ml). Ionomycin caused a rapid increase in $[Ca^{2+}]i$ in a dose-dependent manner,but TPA(below 200nM) did not in this system. When ionomycin and TPA were added together,$[Ca^{2+}]i$ was dependent on the concentration of the former. It is concluded that synergism between Ca^{2+}mobilization and C kinase activation is observed for platelet aggregation as well as release reaction.

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SYNERGISM OF G_i-DISSOCIATION AND PROTEIN KINASE C STIMULATION IN PLATELET ACTIVATION.

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Epinephrine or UK 14304 (a specific α_2-adrenoceptor agonist) synergizes with phorbol esters (phorbol 12,13-dibutyrate, PdBu) or bioactive diacylglycerols (sn-1,2-dioctanoylglycerol, DiC_8) to induce aggregation and ATP-secretion of platelets. The effect on aggregation is more pronounced than on secretion, and it is observed in aspirinized platelet-rich plasma or suspensions of washed platelets containing ADP-scavengers. No prior shape change is found. In the presence of epinephrine, DiC_8 induces reversible aggregation and PdBu evokes irreversible aggregation that correlates with the effects on protein phosphorylation. Epinephrine and UK 14304 neither induce nor enhance the phosphorylation of myosin light chain (20kDa), the substrate of protein kinase C (47kDa), or a 38kDa protein evoked by DiC_8 or PdBu. Epinephrine does not cause stimulation of phospholipase C as reflected by the production of inositol mono-, bis- and tris-phosphate or phosphatidic acid. Even under conditions of maximal aggregation induced by epinephrine plus PdBu, formation of ^{32}P-phosphatidic acid is not observed. The synergistic action of epinephrine and PdBu does not depend on extracellular Ca^{2+}. Primary aggregation induced by epinephrine, but not platelet aggregation induced by PdBu plus epinephrine, is inhibited by high intracellular concentrations of the calcium chelator quin2. Prostacyclin prevents platelet aggregation but does not affect protein phosphorylation induced by PdBu plus epinephrine.

The experiments indicate that α_2-adrenoceptor agonists may induce primary aggregation by a mechanism involving release of membrane-bound Ca^{2+}. The synergism with protein kinase C is, however, caused by a mechanism that occurs distally to protein phosphorylation and is not related to phospholipase C activation and Ca^{2+}-fluxes across the plasma membrane or in the cytosol. Evidence is presented supporting the view that this mechanism might be related to the dissociation of G_i caused by α_2-adrenoceptor activation.

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REGULATION OF PLATELET cAMP FORMATION BY PROTEIN KINASE C.

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Exogenous synthetic 1,2-diacylglycerols (e.g. 1,2-dioctanoylglycerol, DiC_8) and 4β Phorbol esters (e.g. phorbol myristate acetate, PMA) routinely are used to probe the effects of protein Kinase C (PKC) on cellular responsiveness. Such agents act either independently or synergistically with elevated $[Ca^{2+}]i$ to induce platelet activation, but also inhibit agonist-induced inositol lipid metabolism and Ca^{2+} flux. These findings led to the concept that activated PKC can function as a bi-directional regulator of platelet reactivity. Therefore, DiC_8 and PMA were utilized to examine the effects of activated PKC on receptor-mediated stimulation and inhibition of adenylate cyclase, as monitored by cAMP accumulation. All studies were performed using intact human platelets in a modified Tyrodes solution, and cAMP was quantified by radioimmunoassay. Pretreatment (2 min.; 37°C) of platelets with PMA ($\leq$ 300 nM) but not DiC_8 (200 μM) attenuated the elevation of platelet cAMP content evoked by PGD_2 ($\leq$ 300 nM) but not by PGE_1 ($\leq$ 300 nM), PGI_2 ($\leq$ 100 nM) or adenosine ($<$ 100 μM).

These effects of PMA were unaffected by ADP scavengers, by Flurbiprofen (10 μM) or by cAMP phosphodiesterase inhibitors (IBMX, 1 mM) but were abolished by the PKC inhibitor Staurosporine (STP, 100 nM). In contrast, DiC_8 (200 μM), but not PMA ($\leq$ 300 nM), reduced the inhibitory effect of adrenaline (5 μM) on PGE_1 (300 nM)-induced cAMP formation. This effect of DiC_8 was unaltered by STP (100 nM). Selective inhibition of PGD_2-induced cAMP formation by PMA most probably can be attributed to PKC catalysed phosphorylation of the DP receptor. Reduction of the inhibitory effect of adrenaline by DiC_8 could occur via an action at the α_2 adrenoreceptor or Ni. These differential effects of PMA and DiC_8 may result from differences in their distribution or efficacy, or to heterogeneity of platelet PKC.

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ROLE OF GUANINE NUCLEOTIDES IN Ca^{2+} - DEPENDENT LYSOSOMAL SECRETION FROM ELECTROPERMEABILISED PLATELETS.

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Previous studies have shown that the maximal extent of Ca^{2+} dependent secretion of β-N-acetylglucosaminidase (β-N-AcGlc) from electropermeabilised human platelets can be enhanced by addition of thrombin or of 1-oleyl-2-acetylglycerol or 12-O-tetradecanoyl phorbol-13-acetate without a significant alteration in the EC_{50} for Ca^{2+}. (Knight et al. Europ. J. Biochem.,143,437 (1984)). We have found a similar Ca^{2+} dependent increase in the maximal extent of β-N-AcGlc and β-galactosidase secretion on addition of metabolically stable analogues of GTP (GTPγS and GppNHp) in the absence of thrombin or of GTP added in the presence of a non-saturating concentration of thrombin. The EC_{50} values for GTP and GppNHp do not differ significantly for β-N-AcGlc and ^{3}H-5HT secretion, but GTPγS is significantly more effective for ^{3}H-5HT secretion.

	EC_{50}(μM) for secretion of:	
	^{3}H-5HT	β-N-AcGlc
GTPγS (0.8μM Ca^{2+})	6.1 ± 0.8	17.0 ± 1.7
GppNHp (0.8μM Ca^{2+})	26.8 ± 5.4	31.3 ± 2.8
GTP (0.5μM Ca^{2+},2.5μM thrombin)	6.4 ± 0.8	7.5 ± 0.6

The time course of β-N-AcGlc secretion induced by GTPγS shows a significant delay as compared with that induced by thrombin + Ca^{2+}. No significant differences could be detected between the properties of β-N-AcGlc or β-galactosidase secretion in this system. The results are consistent with involvement of a GTP binding protein (N_p) in receptor-phospholipase C coupling mediating lysosomal secretion, but provide no indication that an additional protein of this type (N_e) is involved as has been proposed for lysosomal secretion from neutrophils. We have thus far failed to find evidence for heterogeneity in lysosomal secretion in this system (supported by SERC and Ciba-Geigy).