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ACTION OF GTPγS [GUANOSINE 5'-O-(3-THIOPHOSPHATE)] ON SAPONIN-PERMEABILISED PLATELETS: INVOLVEMENT OF 'G' PROTEINS IN PLATELET ACTIVATION.
K. S. Authi, B. J. Evenden and N. Crawford.
Department of Biochemistry, Royal College of Surgeons of England, London, U.K.

Certain ligand-receptor interactions at cell surfaces lead to the phospholipase-C (PLC) hydrolysis of phosphatidyl inositol (4.5) bisphosphate (PIP_2). The products serve as intracellular second messengers, e.g. inositol (1.4.5) trisphosphate (IP_3) releases Ca^{2+} from intracellular stores and diacylglycerol activates protein kinase-C. From studies using GTP and analogues (e.g. GTPγS) there is evidence of a key role for a guanine nucleotide binding protein(s) as a link between receptors and PIP_2 hydrolysis. We report the actions of GTPγS on washed human platelets permeabilised with saponin (12-14 μg/ml) to allow penetration of low MWt polar substances. The responses to GTPγS are dose dependent (range 9-60 μM) and at 60 μM the agent induces shape change, aggregation and the secretion of 50% of previously incorporated [^{14}C]-5HT. No effect of GTPγS is seen with intact cells. Shape change occurs 25-30 sec after GTPγS; aggregation and secretion is complete after 3 min. When GTP was used (up to 135 μM) with similarly permeabilised platelets no responses were initiated. Phosphatidylinositol turnover was monitored using ^{32}P-labelling before permeabilisation. The addition of 90 μM GTPγS resulted in a 143 $\pm$ 23% (n=4) increase in ^{32}P-phosphatidic acid (PA) with respect to the basal levels of "saponised control" cells. These findings suggest that GTPγS stimulates PLC activity through a 'G' protein interaction. The GDP analogue (GDPβS) produced no activation responses in saponised platelets but inhibited responses induced by GTPγS in a dose dependent manner (0-480 μM, max inhibition 480 μM). At 960 μM, GDPβS totally inhibited aggregation and secretion initiated by low doses of thrombin (0.1 U/ml) and collagen (1 μg/ml). Identical inhibition by GDPβS of thrombin and collagen-induced activation of intact platelets was observed indicating membrane penetration of this analogue. Shape change effects were not inhibited by GDPβS. The inhibitory effects of GDPβS towards thrombin and collagen induced secretion could be progressively overcome at higher doses of thrombin (0.2 U/ml - 2 U/ml) and collagen (5 μg/ml - 60 μg/ml) suggesting that at higher concentrations these agonists may exert effects through 'G' protein-independent mechanisms.

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THROMBIN-INDUCED RELEASE OF INTRA-PLATELET CA^{2+} STORES IS INHIBITED BY PROSTACYCLIN, BUT GTP- AND IP3-INDUCED RELEASE IS UNAFFECTED.
Michael F. Crouch, Roger D. Nolan and Eduardo G. Lapetina. Molecular Biology Department, The Wellcome Research Laboratories, Research Triangle Park, N.C. 27709, U.S.A.

Alpha-thrombin induced the release of internal Ca^{2+} stores and the influx of Ca^{2+} in human platelets, as measured by quin-2 fluorescence. This was accompanied by a stimulated formation of inositol phosphates and phosphatidic acid. The Ca^{2+} responses were inhibited almost totally by pretreatment of cells with prostacyclin (PGI_2). Epinephrine was able to restore the influx of Ca^{2+} from the external medium, but not the alpha-thrombin-induced release of internal Ca^{2+} stores. This was despite epinephrine restoring phosphatidic acid formation and, at least partially, the generation of inositol trisphosphate (IP3). This suggested that PGI_2 was inhibiting the actions of IP3 in inducing release of Ca^{2+} from the dense tubular system. Since the effects of PGI_2 are thought to be mediated by formation of cAMP, we examined whether cAMP could modulate the release of $^{45}Ca^{2+}$ induced by IP3 from permeabilized platelets. IP3 induced about a 30% release of cellular $^{45}Ca^{2+}$ over a 4 min period. However, neither pretreatment of cells with PGI_2 nor the direct application of dibutyryl cAMP had any effect on the IP3-stimulated $^{45}Ca^{2+}$ release. GTP, which released about 10% of total cell $^{45}Ca^{2+}$, also was not affected by these agents. These results suggest either that permeabilization of platelets dilutes cytoplasmic components which are necessary for cAMP action, or that PGI_2 is inhibiting the release of Ca^{2+} stores induced by thrombin, presumably via IP3, by a mechanism which is separate to the elevation of intracellular cAMP levels.

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POSSIBLE RELATIONSHIP BETWEEN THE 23-kDa PHOSPHOPROTEIN AND THE IP_3 -INDUCED Ca^{2+} RELEASE IN HUMAN PLATELETS.
J. Enouf(1), R. Bredoux (1), . Giraud (2), N. Bourdeau (1) and S. Levy-Toledano (1). U-150 INSERM, UA 334 CNRS, Hôpital Lariboisière, Paris, France (1) and Université Paris-Sud, Orsay, France(2).

The regulation of Ca^{2+} concentration in human platelets involves intracellular membranes i.e. dense tubular system (DTS). Agonist-induced platelet activation generates inositol 1,4,5 trisphosphate (IP_3) which is responsible for Ca^{2+} mobilization from DTS. However, its mechanism of action is still unknown. cAMP has been shown to regulate Ca^{2+} transport by isolated membrane vesicles. This effect was correlated with the phosphorylation of a 23 kDa protein. We investigated whether this phosphorylation could play a role in the mechanism of IP_3-induced Ca^{2+} release.

We isolated a membrane fraction enriched in intracellular membranes, which actively sequesters Ca^{2+}. The Ca^{2+} uptake was mediated by a characterized (Ca^{2+} + Mg^{2+})-ATPase of a molecular weight 120 kDa. As well, the characterization of the 23-kDa protein phosphorylation by the catalytic subunit of the cAMP dependent protein kinase (C. Sub.) has been achieved.

IP_3-induced Ca^{2+} release was tested on our membrane preparations. The transient effect was maximal at one minute and a dose-response curve was obtained.

The cAMP dependent phosphorylation of the 23-kDa protein increased the Ca^{2+} liberation induced by IP_3 by two fold whatever the IP_3 concentration. The addition on the protein kinase inhibitor inhibited the IP_3-induced Ca^{2+} release.

The effect of IP_3 on the cAMP-mediated phosphorylation of the 23-kDa protein has been examinated.A dose dependent stimuulation of the 23-kDa protein phosphorylation in the presence of C. Sub. was initiated by IP_3. The maximal effect was observed after 1-2 min and obtained with an IP_3 concentration similar to that producing the maximal calcium release. The stimulation of the phosphorylation by IP_3 was detected in the absence of Ca^{2+} and in the presence of phosphatase inhibitors.

Therefore, we suggest a possible correlation between cAMP dependent phosphorylation of the 23-kDa protein and the IP_3-induced Ca^{2+} release in human platelet membrane vesicles.

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Phosphoinositide metabolism in resting and thrombin-stimulated human platelets: Evidence for metabolic homogeneity.
O.-B. Tysnes, A.J.M. Verhoeven, G.M. Aarbakke and H. Holmsen.
Department of Biochemistry, University of Bergen,
Årstadveien 19, N-5000 Bergen.

On the basis of differences in specific radioactivity (SA), separate pools of phosphoinositides have recently been proposed in platelets. Human platelets were labelled for 60 min with [^{32}P]P_i and subsequently transferred to a phosphate- and Ca^{2+}-free Tyrode's solution by gel-filtration. Thereafter, the platelets were either incubated at 37°C for 120 min, a condition which induces increase in specific labelling of the diester phosphate of phosphatitylinositol (PI), or stimulated with 0.5 U/ml of thrombin. The changes in SA of both diester and monoester phosphates of the phosphoinositides were detrmined. Immediately after the gel filtration, the SA of the diester phosphate of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) were both similar to that of PI and amounted to 4% of the SA of the monoester groups of PIP and PIP_2. Whereas the SA of the monoester phosphate essentially remained constant and the same for PIP and PIP_2 during the entire incubation, the SA of their diester phosphates increased gradually in parallel to that of PI, and reached 20% of the monoster groups after 120 min. The effect of thrombin was studied at 15, 60 and 180 sec after the addition. The absolute radioactivity of both diester and monoester phosphates of all phosphoinositides increased conciderably after an initial decrease. However, for the monoester groups, the changes in radioactivity were parallelled by the changes in mass for both PIP and PIP_2. Thrombin therefore induced no changes in SA of the monoester phosphates. In contrast, the SA of the diester phosphates increased 5-fold and remained similar for all three phosphoinositides during the 180 sec of stimulation.
In conclusion, our results demonstrate close metabolic equilibrium between all three phosphoinositides.
Thrombin-induced changes in SA of PIP and PIP_2 are purely secondary to changes in specific labelling of the diester phosphate.

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