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HIGH CONCENTRATIONS OF EXOGENOUS ARACHIDONATE INHIBIT CALCIUM MOBILIZATION IN PLATELETS. M.A. Kowalska, A. K. Rao, J. Disa, Thrombosis Research Center and Department of Medicine, Temple University, Philadelphia, PA, U.S.A.

Exogenous arachidonic acid (AA) induces platelet aggregation and secretion which are inhibited at higher AA concentrations. To define the mechanisms for platelet inhibition at high AA, we studied the effect of its sodium salt on cytoplasmic ionized calcium concentration $[Ca^{2+}]_i$, a key modulator of several platelet responses. In platelets suspended in HEPES buffer containing no albumin, peak aggregation and secretion occurred at 2-5 μM AA with partial inhibition above 10-15 μM AA and complete inhibition around 25-50 μM AA. In platelets loaded with quin2, $[Ca^{2+}]_i$ rose, in presence of 1 mM external Ca^{2+} , from basal levels of 70-80 nM to peak of 300-500 nM at 2-5 μM AA; this was followed by inhibition to basal levels at 25-50 μM AA. At these AA concentrations there was no cell lysis. Thromboxane B_2 production, measured using a radioimmunoassay, was not inhibited even at 25 μM AA. Elevated cellular levels of cAMP inhibit platelet responses including Ca^{2+} signals and were therefore measured using a radioimmunoassay. Platelet cAMP levels rose, in the presence of theophylline (7mM), from basal levels of 3.4 pmol/10⁶ platelets to 5.5 at 5 μM AA and to 6.8 pmol/10⁶ platelets at 50 μM AA; Ca^{2+} signals, aggregation, and secretion were inhibited with doubling of cAMP levels. On incubation of platelets with adenylate cyclase inhibitor, 2',5'-dideoxyadenosine (DDA, 200 μM , 2 min) there was enhancement of peak $[Ca^{2+}]_i$ and aggregation noted with 15 μM AA; at 25 μM AA peak $[Ca^{2+}]_i$ rose from 126 nM to 205 nM and aggregation was restored. Incubation with SQ 22,536 (500 μM , 5min), another adenylate cyclase inhibitor, also attenuated the inhibition by high AA levels. Treatment of platelets with aspirin or BW 755C, a combined lipooxygenase/cyclooxygenase inhibitor, did not prevent the inhibition by high AA levels of aggregation and Ca^{2+} responses induced by thromboxane analog, U46619. In conclusion, high AA concentrations inhibit cytoplasmic Ca^{2+} mobilization in platelets which is related to elevation of platelet cAMP through stimulation of adenylate cyclase. We propose that high AA levels inhibit aggregation and secretion by modulating cytoplasmic levels of Ca^{2+} and cAMP, two major messenger molecules in platelets.

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REGULATION OF RECEPTOR-OPERATED Ca^{2+} CHANNEL OPENING IN HUMAN PLATELETS. Katrina J. Moffat and D. Euan MacIntyre*, Department of Pharmacology, University of Glasgow, Scotland and *Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A.

Agonist-induced elevation of the platelet intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), as monitored using quin2, is not electrically mediated and is attenuated by removal of extracellular Ca^{2+} and by lanthanides (e.g. Gd^{3+}). Collectively these data suggest that elevation of $[Ca^{2+}]_i$ in platelets derives in part via influx of external Ca^{2+} presumably through a receptor-operated Ca^{2+} channel (ROC). Hallam & Rink (FEBS Lett. 186: 175: 1985) showed that Mn^{2+} also enters platelets via these ROC. To investigate the possible regulatory mechanisms that govern ROC status, we utilized quin2-labelled human platelets suspended in a Ca^{2+} -free HEPES buffered Tyrodes solution, and monitored agonist-induced Mn^{2+} -mediated quenching of quin2 fluorescence as an index of ROC opening.

Thrombin (Th, 0.01-1 U/ml), Vasopressin (VP, 10-1000 nM) and the TxA_2 -mimetic, EP171 (1-100 nM) all induced ROC opening which occurred rapidly ($< 30s$), was maximal within 30-60s and thereafter declined. Gd^{3+} (≤ 2 mM) markedly impaired this Mn^{2+} -mediated quenching of quin2 fluorescence induced by all 3 agonists. The adenylate cyclase stimulant PCD_2 (3-3000 nM) and the guanylate cyclase stimulant sodium nitroprusside (0.01-10 μM) impaired ROC opening induced by Th (0.5 U/ml), VP (100 nM) and EP171 (25 nM) whether added to platelets $\leq 120s$ before or 30s after the agonists. In contrast, agents that selectively antagonize, at the receptor level, the effects of VP (e.g. $d(CH_2)_5$ Tyr Me AVP, 10 μM) or EP171 (e.g. EP092, 250 nM), or that inhibit the action of Th (e.g. Hirudin 1 U/ml) only impaired ROC opening when added to platelets simultaneously with or before the agonist. These results indicate that, although initiated by agonist-receptor interaction, maintenance of the open state of ROC in human platelets does not require continued receptor occupancy or activation by agonist. Moreover, besides acting to impair the transduction processes initiated following occupancy by agonist of platelet V_1 , TP and Thrombin receptors, cAMP- and cGMP-dependent reactions also can terminate or otherwise limit opening of ROC.

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THE DEPENDENCE OF PLATELET ATP SECRETION IN RESPONSE TO VARIOUS AGONISTS ON Ca^{2+} MOBILIZATION AND AGGREGATION IN QUIN 2 LOADED PLATELETS. M.J. Powling and R.M. Hardisty, Department of Haematology and Oncology, Institute of Child Health and Hospital for Sick Children, London, England.

Thrombin (0.05 and 0.5 u/ml), ADP (10 μM), PAF (30 nM), vasopressin (VP, 0.018 u/ml), arachidonate (AA, 10 μM), U46619 (0.125 $\mu g/ml$) and A23187 (200 nM) induce aggregation, ATP secretion, TxB_2 generation and a rapid elevation of intracellular calcium (Ca^{2+}) concentration provided the medium contains 1 mM extracellular Ca^{2+} . Phorbol ester (TPA) induces aggregation and secretion with no alteration in $[Ca^{2+}]_i$. Pre-incubation with Fab fragments of M148, a monoclonal antibody against GPIIb/IIIa, completely abolished aggregation in response to all agonists without affecting Ca^{2+} flux. ADP and A23187-induced secretion was completely abolished by M148 Fab, while that induced by VP, PAF, U46619, AA, thrombin and TPA was inhibited by 70%, 70%, 30%, 25%, 25% and 10% respectively.

In the presence of 1 mM EGTA ($Ca^{2+} < 100$ nM), aggregation was again abolished and the Ca^{2+} fluxes were about 10-20% of those in the presence of 1 mM Ca^{2+} . ADP and A23187 again induced no secretion, but 0.05 u/ml thrombin, PAF, VP, U46619, AA, 0.5 u/ml thrombin and TPA induced respectively 6%, 8%, 12%, 30%, 40%, 57% and 85% of control (1 mM Ca^{2+}) levels. Addition of M148 Fab and EGTA together had no greater effect than EGTA alone.

At the concentrations used, ADP and A23187 are thus completely dependent on aggregation for the induction of ATP secretion, while TPA and thrombin (0.5 u/ml) can act largely independently of both aggregation and changes in $[Ca^{2+}]_i$. Low concentrations of thrombin induce secretion in the absence of aggregation provided the stimulated $[Ca^{2+}]_i$ is sufficiently high (ie greater than that seen with < 100 nM Ca^{2+}). VP and PAF appear to be more dependent on aggregation for their secretory responses, while U46619 and AA are more dependent on stimulated $[Ca^{2+}]_i$.

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A MONOCLONAL ANTIBODY (PL/IM 430) THAT BLOCKS THE ACTIVE TRANSLLOCATION OF Ca^{2+} INTO HUMAN PLATELET INTRACELLULAR MEMBRANE (ER) VESICLES. N. Hack, J.M. Wilkinson and N. Crawford, Department of Biochemistry, Royal College of Surgeons of England, London, U.K.

In earlier studies [1] we identified a number of important biological properties associated with highly purified human platelet intracellular membrane (ER), isolated by continuous Ca^{2+} flow electrophoresis. These included a high affinity Ca^{2+} -Mg²⁺ ATPase and protein phosphorylation both of which are involved in the active uptake of Ca^{2+} into ER vesicles. The stored Ca^{2+} could be released with inositol(1,4,5)trisphosphate, (IP_3), (approx. 50% release in 30 s 1/2 max. for release - 0.25 μM IP_3) [2]. To probe the structure-function relationship of proteins in these ER vesicles, a panel of monoclonal antibodies (Mabs) has been raised, using the ER membrane preparation as immunogen. Four of these Mabs recognise a single 100 kDa polypeptide by immunoblotting. This protein is present in platelet membranes and can also be identified in cultured human monocyte, macrophage and endothelial cell lines. None of the Mabs showed any significant effect upon the ER membrane Ca^{2+} -Mg²⁺ ATPase activity but one, PL/IM 430 (of IgG1 subclass), inhibited the Ca^{2+} sequestration by the vesicles significantly (approx. 70% inhibition at 10 μM IgG). This inhibition was independent of the ATP concentration over a range of 0.2 mM ATP, but was dose-dependent for external free Ca^{2+} between 30-300 nM Ca^{2+} , giving maximum inhibition at 300 nM Ca^{2+} with 10 μM IgG. Binding of the antibody substantially lowers the V_{max} for Ca^{2+} uptake but is without effect upon the K_m . PL/IM 430 therefore appears to recognise a 100 kDa polypeptide closely involved with Ca^{2+} translocation but at a site which is without effect upon the Ca^{2+} -Mg²⁺ ATPase associated with the Ca^{2+} pump.

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[1] Hack, N., Croset, M. and Crawford, N. (1986) Biochem. J. 233, 661-668.

[2] Authi, K. S. and Crawford, N. (1985) Biochem. J. 230, 247-253.