SYNERGISTIC INTERACTION OF EPINEPHRINE AND CALCIUM IONOPHORE IN PLATELET AGGREGATION

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Background: Platelets play a key role in haemostasis. Human Platelets contain α_2 adrenergic receptors, which are coupled with guanine nucleotide proteins (G proteins). The platelet activation involves a number of receptors for agonists. It has also been shown that most of the agonists act in synergy and potentiate the effects of each other. The present experimental study was designed to study the potentiation of epinephrine on human platelets by calcium ionophore A23187 and the possible role of calcium in platelet aggregation as a second messenger. **Methods:** Study was carried out at Department of Biological Sciences Aga Khan University, Karachi. Blood samples from healthy volunteers were collected; Platelet aggregation was measured using Dual channel Lumi Aggregometer. The chemicals used include epinephrine, calcium ionophore A23187, yohimbine, diltiazem, verapamil and S Nitrosoacetylpenicillamin (SNAP).**Results:** Epinephrine at low concentrations (0.01 - 0.2 μM) and/or A23187 (0.1-0.5 μM) itself did not produce platelet aggregation. However, when added together, a marked potentiation of platelet aggregation was observed. This synergistic effect was inhibited by α_2-receptor blocker yohimbine; (IC_{50} = 0.05 μM) showing that the response is receptor mediated. To find out the molecular basis of this potentiation, we used SNAP, a nitric oxide donor and Ca^{++} channel blockers,i.e. diltiazem and verapamil. The SNAP, diltiazem and verapamil inhibited the platelet aggregation induced by A23187 and epinephrine with IC_{50} value of 0.5 μM, 50μM and 22μM respectively. **Conclusion:** The results of the study suggest that epinephrine and calcium ionophore act synergistically and Ca^{++} plays an important role in this synergistic interaction. While calcium channels blocking drugs diltiazem and verapamil inhibit this synergism.

Key Words: Platelet aggregation, Epinephrine, calcium ionophore A23187, yohimbine, diltiazem, verapamil and SNAP.

INTRODUCTION

Platelets are known to be engaged in a variety of biochemical and molecular activities designed to prevent haemorrhage and maintain vascular integrity. To accomplish these tasks, platelets have surface receptors that can bind adhesive glycoproteins (GP) of various types and thus promote platelet adhesiveness, aggregation and release reaction. The platelet activation process involves a number of receptors for agnosits such as adenosine diphosphate (ADP), epinephrine, thrombin, collagen, fibrinogen, thromboxame A2, (TXA2) and platelet activating factor (PAF). It also involves several signal transduction pathways, including phosphoinositide metabolism, arachidonic acid release and conversion into thromboxame A2, calcium mobilization and phosphorylation of a number of different target proteins. Many platelet agonists, like thrombin, ADP, PAF, epinephrine and 5 hydroxytryptamin (5 HT) initiate platelet activation by binding to transmembrane receptors on platelets coupled with guanosine triphosphate (GTP) binding proteins (G proteins). The G proteins mediate a variety of cellular processes by activating different effector molecules, like adenylyl cyclase, phospholipase C (PLC) or ion channels[1]. Epinephrine a very important hormone of the adrenal gland, can influence platelet aggregation like other agonists. Epinephrine receptors have been classified as alpha and beta with respective subtypes α 1. α 2, β1 and β 2. Human platelets contain both α 1, and α 2, adrenoceptors. The α 2 adrenoceptor is identified as being primarily responsible for mediating the response to natural agonists[2,3]. The receptor (α_2) is a G.inhibitory (Gi) protein linked receptor that contains seven membrane spanning hydrophobic domains, an extracellular binding site and a series of cytoplasmic binding loops that are the

sites of interaction with Gi proteins in the cytoplasm[4]. It is also known that activation of α_2 adrenergic receptors in human platelets inhibit the adenyl cyclase system through coupling to a Gi protein[5]. It is considered that inhibition of adenyl cyclase system is not sufficient to cause platelet aggregation, but may be sufficient to amplify the activation induced by other agonists[6]. Indeed, the signal processing mechanisms which mediate the platelet stimulating effect of epinephrine have not so far been clarified despite extensive research [7-11].

Calcium ionophore A23187 is thought to activate cellular phospholipases and thus causes calcium entry into the cell from the extracellular fluid (ECF). It has also been shown that most of the agonists act in synergy and potentiate the effect of each other[12]. The phenomenon of agonist synergism is very important physiologically and has been demonstrated in many pairs of agonists[13-18]. The possible mechanism of this synergistic action is by raising cytoplalsmic concentration of Ca^{++}.The first agonist or initial stimulus "primes" the platelets for an augmented response to a second agonist[19]. The cytoplasmic Ca^{++} concentration can be increased by two ways, either by causing an influx of Ca^{++} into the cell from ECF or by causing a release of Ca^{++} from intracellular stores. The role of other effectors and second messengers is not well understood. The present study was designed to study the potentiation of epinephrine by A23187 on human platelets and to find out the molecular basis of this potentiation.

MATERIAL AND METHODS

This experimental study was conducted on 200 samples of platelets at Department of Biological Sciences Aga Khan University, Karachi. Epinephrine, calcium ionophore A23187, Yohimbine, Diltiazem, Verapamil were obtained from Sigma chemicals company St Louis, USA and S. Nitroso Acetyl Penicillamin (SNAP) from Alexis LC – Labs (UK).

Blood was taken by venipuncture from healthy human volunteers reported to be free of medication for last two weeks. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) in plastic centrifuge tubes and centrifuged at 1100 revolution per minute (r.p.m) for 15 minutes at room temperature to obtain platelet rich plasma (PRP). The PRP was removed carefully with micropipette into the cuvettes and placed at room temperature. The remaining blood was centrifuged again at 3000 r.p.m for 5 minutes to obtain platelet poor plasma (PPP).

Platelet aggregation was measured with platelet aggregometer (Model 440, Chronolog Corporation-USA) using the technique described originally by Born [20]. The changes in optical density were recorded on omniscribe chart recorder. Temperature (37Ċ), stirrer speed (1100 r.p.m) and speed of chart recorder (25 mm per minute) were kept constant. The cuvette containing 500 µl PPP was placed in PPP reference well. The cuvettes containing PRP were placed in the incubation wells. At the time of testing each cuvette contained 450 µl PRP and a Teflon coated stirrer bar. The final volume of PRP under test was made 500 µl by adding 50 µl of the test drug. The resulting aggregation response was recorded for 5 minutes after challenge by the agonists. Aggregation was induced with epinephrine and A23187 and subthreshold concentration determined for each agonist. To determine the synergistic effect of epinephrine and A23187, we added subthreshold concentration of each agonist together. Successive samples of PRP were tested with different concentrations of drug. Once, the antiplatelet activity of various inhibitors (Yohimbine, Diltiazem, Verapamil, SNAP) against agonists was determined, the dose response curves were constructed to calculate IC 50 (Half maximal inhibitory concentration)values.

RESULTS

The Platelet aggregation induced by different concentrations of epinephrine (0.1, 0.2, 0.5, 1.0, 5.0 µM) was recorded. The results indicated 0.2uM as the subthreshold concentration that does not induce optimal platelet aggregation. The platelet aggregation induced by different concentrations of A23187 (0.25, 0.50, 1.0, 2.0, 5.0 µM) was also recorded. The results indicated, 0.5 uM as the subthreshold concentration for A23187. To establish the synergistic effect of epinephrine and A23187, the PRP was challenged with subthreshold concentration of epinephrine (0.2 µM) and subthreshold concentration of A23187 (0.5 µM) simultaneously and an optimal platelet aggregation response

(35% intensity of aggregation) was observed. The results shown in Fig-1 manifest that optimal platelet aggregation is only recorded when platelets are challenged with the subthreshold concentrations of epinephrine and A23187 together. While decreasing the concentration of any one of the agonist and keeping constant the concentration of the other agonist, does not produce optimal platelet aggregation, thus establishing the synergistic effect of epinephrine and A23187.

Fig-1a: Percentage platelet aggregation responses induced by synergistic effect of subthreshold concentration (0.5μM) of A-23187 and different subthreshold concentrations (0.005, 0.01, 0.05, 0.1, 0.2μM) of Epinephrine

Fig-1b: Percentage platelet aggregation responses induced by synergistic effect of subthreshold concentrations (0.2μM) of epinephrine and different subthreshold concentrations (0.6, 0.12, 0.25, 0.5 μM) of calcium ionophore A-23187.

When PRP was pretreated with different concentrations, (0.01, 0.05, 0.1 μM) of Yohimbine an α2 adrenergic receptor blocker and then the platelet aggregation was induced by adding simultaneously the subthreshold concentration of epinephrine (0.2 μM) and A23187 (0.5 μM), there was inhibition of platelet aggregation in a dose dependent manner (Fig-2), IC50 value for the Yohimbine was calculated to be 0.05 μM. Similarly when the PRP was pretreated with different concentrations (10,20,40,60 μM) of calcium channel blocker, Verapamil and different concentrations (10,20,40,60,80,100 μM) of Diltiazem, another calcium channel blocker, it inhibited the platelet aggregation induced by subthreshold concentration of epinephirine (0.2 μM) and subthreshold concentration (0.5 μM) of A23187 in dose dependent manner . A dose response curve shown in Fig-3 manifests a dose dependent inhibitory effect of both the Verapamil and Diltiazem. The IC50 values of Verapamil and Diltiazem as calculated from the curve are 22 μM and 50 μM respectively. When PRP was pretreated with different concentrations (0.1,0.2,0.4 μM) of SNAP, a nitric oxide (NO) donor and then the platelet aggregation was induced by adding together the subthreshold concentrations of epinephrine (0.2 μM) and A23187 (0.5 μM, the platelet aggregation was inhibited in a dose dependent manner Fig-4. Dose response curve was constructed and IC50 value was calculated to be 0.5μM.

Fig -2 Effect of different concentrations (0.1, 0.05, 0.01μM) of Yohimbine (an α_2 adrenergic receptor blocker) on platelet aggregation induced by subthreshold concentrations of Epinephrine (0.2μM) and A-23187 (0.5μM) shown as control

DISCUSSION

The results of the present study have shown that there is potentiation of epinephrine effects by A23187 in human platelets. Our results also indicate that the platelet activation induced by epinephrine is mediated through α_2 adrenergic receptors and ther is a definite role of Ca^{++} in this synergism. Furthermore the results also exhibit that calcium channel blocking agents inhibit the platelet aggregation induced as a result of epinephrine, A23187 synergism. While this synergism was also inhibited by SNAP (a nitric oxide donor).

Fig-3: Dose response inhibitory effect of verapamil and diltiazem on platelet aggregation induced by subthreshold concentration of epinephrine (0.2μM) and A23187 (0.5μM). Data is Mean ±SEM (n=5)

Fig-4: Effect of different concentrations (0.4, 0.2, 0.1μM) of SNAP (nitric Oxide donor) on platelet aggregation induced by subthreshold concentrations of epinephrine (0.2μM) and A23187 (0.5 μM) shown as control

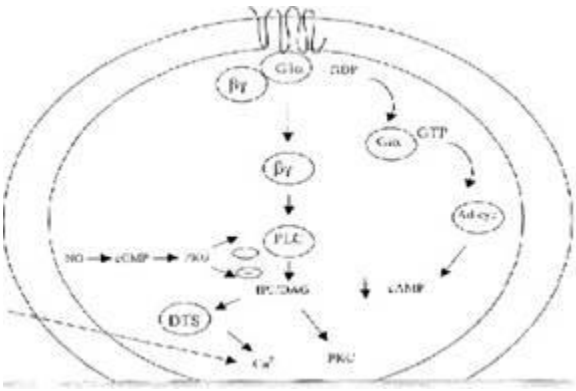

Fig -5: Proposed platelet model depicting the role of Gi protein and calcium channels during coactivation by epinephrine and A-23187. Giβγ subunit activates PLC, forming Inositol triphosphate (IP_3) and diacylglycerol (DAG).IP_3 causing release of Ca^{++} from dense tubular system (DTS) while A23187 promoting Ca^{++} entry into the cytoplasm from outside. NO from exogenously added SNAP (NO donor) inhibits platelet aggregation through production of cGMP. The cGMP activates protein kinase G (PKG) which inhibits PLC induced IP_3 formation.

In our proposed platelet model, (Fig-5) epinephrine and A23187 synergism raises cytosllic Ca^{++} concentration on one hand by release from intracellular stores (dense tubular system) and on the other hand by increased influx from the extracellular fluid. Similar mechanism of agonist syndergism is known among other agonists also and is considered to occur due to activation of calcium signaling cascade. Recent studies have shown that βχ subunits of activated Gi protein can also activate phospholipase C (PLC)[21-22]. Activation of PLC pathway leads to an increase in cytosolic Ca^{++} due to its release from internal stores i.e dense tubular system (DTS) by inositol triphosphate (IP3) or through store depletd calcium influx[23-24]. The platelet cell membrane has limited permeability to calcium but is penetrated by several channels capable of permitting calcium influx. The calcium channels are protein macromolecules. Different types of calcium channels have been identified. Based on the stimulus required for opening of channels, there are voltage dependent calcium channels (VDCC), receptor operated channels (activated by binding of a chemical ligand to receptor) and second messenger operated channels (cAMP dependent channels). Most of the calcium influx during platelet activation results from passage through receptor operated calcium channels[12]. But the reagents that block VDCC, like Verapmil and Diltiazem, can also prevent elevation of intracellular calcium induced by several agonists [12-14].

To study the molecular basis of this potentiation, SNAP, was used that inhibited the platelet aggregation induced by the synergistic interaction of subthresthold concentrations of epinephrine and A23187. These results suggest that this synergism is sensitive to NO generation. Platelets contain an abundance of cAMP and cGMP dependent protein kinases which are activated by NO and inhibit PLC induced IP_3 and thramboxane receptors thus inhibiting platelet aggregation[24,25]. The intracellular signaling involved in this synergism is likely to be mediated through stimulation of PLC of βχ subunits of Gi protein which in turn stimulates IP_3 production and thus mobilinze Ca^{++} from intracellular stores in DTS. The DTS is rich in IP_3 receptors and Ca^{++} is released whenever IP_3 binds to its receptor. Our results are in conformity with the results of another study where the role of NO in inhibiting the platelet aggregation has been claimed[25]. In another recent study [13] it has been suggested that Gi and Gq proteins activation lead to PLC stimulation and Ca^{++} signaling when the synergistic interaction of epinephrine and 5-hydroxytryptamine was studied. They also observed the inhibitory effect of SNAP on this synergism. The results are similar to this study. The results of this study are in agreement with another similar study[18] where potentation of A23187 and epinephrine has been shown. These workers have also shown the inhibitory effect of calcium channel blocker, Diltiazem. But these workers did not explain intracellular signaling involved in this potentiation. In conclusion, our results reveal that potentiation of epinephrine by A23187 involves raised cytosolic Ca^{++} concentration as a result of increased Ca^{++} influx from ECF and simultaneous mobilization of Ca^{++} from intracellular stores by stimulation of PLC by βχ submit of Gi protein.

The study also points to the communication between two different types of receptors that exhibit synergism i.e. Gi protein linked α_2 receptor and calcium channels. In recent days, there is growing evidence for such cross talk between different receptors that leads to platelet aggregations [26-27]

Acknowledgement

This study was supported by Aga Khan University, Karachi. Authors are highly obliged for the provision of chemicals, equipment and logistic support.

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