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Accumulation of the inositol phosphates in thrombin-stimulated, washed rabbit platelets in the presence of lithium

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Experiments with washed rabbit platelets demonstrate that stimulation with a low concentration of thrombin (0.1 unit/ml), that causes maximal aggregation and partial release of amine granule contents, also causes increased accumulation of [^{3}H]inositol-labelled inositol trisphosphate (InsP_3) in the presence of 20mM-Li^+. This concentration of Li^+ was found to inhibit the degradation of inositol phosphates by phosphomonoesterases. This result indicates that phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is degraded early after platelet stimulation with thrombin, although in a previous study we had found no decrease in amount. In the absence of Li^+, the labelling of inositol bisphosphate (InsP_2) increased more rapidly than that of InsP_3, consistent with rapid degradation of InsP_3 by phosphomonoesterase. After 30s the increase in InsP_2 was augmented by Li^+. This increase in InsP_2 could have been due to increased degradation of phosphatidylinositol 4-phosphate or inhibition of breakdown of InsP_2 to Ins$P$ with a lesser inhibition of breakdown of InsP_3 to InsP_2. The effect on InsP_3 and InsP_2 of stimulation of the platelets with 1.0 unit of thrombin/ml was comparable with the effect of the lower concentration of thrombin. Inositol phosphate (Ins$P$) labelling did not increase in response to 0.1 unit of thrombin/ml, but increased when the platelets were stimulated with 1.0 unit of thrombin/ml. Whether the increase in Ins$P$ was due to increased degradation of phosphatidylinositol or a greater rate of breakdown of InsP_2 to Ins$P$ than Ins$P$ to inositol cannot be determined in these experiments. These results indicate that degradation of PtdIns(4,5)P_2 is an early event in platelet activation by thrombin and that formation of inositol phosphates and 1,2-diacylglycerol rather than a decrease in PtdIns(4,5)P_2 may be the important change.

Stimulation of platelets by thrombin has been shown to cause changes in the inositol phospholipids and phosphatidic acid in association with platelet aggregation and the platelet release reaction (Lloyd & Mustard, 1974; reviewed by Rittenhouse, 1983*a*). The relationship of the phospholipid changes to the response of the platelets is not known, although accumulation of PtdA, lysoPtdA or 1,2-diacylglycerol has been suggested to be important in placelet aggregation and the release reaction (Gerrard *et al.*, 1978; Nishizuka, 1983).

In other tissues, degradation of the polyphosphoinositides, particularly PtdIns(4,5)P_2, appears to be linked to receptor activation (Abdel-Latif *et al.*, 1977; Berridge, 1983; Michell *et al.*, 1981; Putney *et al.*, 1983). Recently, in studies of human platelets stimulated with thrombin, Imai *et al.* (1983) have shown an early decrease in the ^{3}H labelling of PtdIns(4,5)P_2 and Rendu *et al.* (1983) showed early decreases in ^{32}P labelling of PtdIns(4,5)P_2. Billah & Lapetina (1982) have demonstrated a rapid decrease in the amount of PtdIns(4,5)P_2 in horse platelets stimulated with 2 units of thrombin/ml. Agranoff *et al.* (1983) showed that ^{32}P-labelled InsP_3, produced by the hydrolysis of PtdIns(4,5)P_2 by phospholipase C,

Abbreviations used: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdA, phosphatidic acid; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; Ins$P$, inositol phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

appeared within 10s of stimulation of human platelets with a high concentration of thrombin, although the data presented were somewhat variable. In addition, Rittenhouse (1983*b*) has demonstrated the presence of phospholipase C activities which degrade the polyphosphoinositides in human platelets. The results indicate an involvement of PtdIns(4,5)P_2 in the response of platelets to thrombin. In contrast with these observations, we found no decrease in the amount of PtdIns(4,5)P_2 in rabbit platelets stimulated with 0.1 unit of thrombin/ml, which causes platelet aggregation and the platelet release reaction. However, stimulation of platelets with 0.3 unit of thrombin/ml caused a decrease in PtdIns(4,5)P_2 (Vickers *et al.*, 1984). The absence of a decrease in the amount of PtdIns(4,5)P_2 could have been due to increased synthesis from PtdIns4*P* which balanced the degradation of PtdIns(4,5)P_2 in the platelets stimulated with the lower concentration of thrombin. In the case of the high thrombin concentration, the synthesis from PtdIns4*P* may not have been sufficient to compensate for degradation.

The appearance of inositol phosphates, as a result of the degradation of the phosphoinositides by phospholipase C, can be used as a sensitive means to detect the degradation of the phosphoinositides (Michell, 1982). However, the inositol phosphates can be degraded by phosphomonoesterase(s) and thus the failure to observe accumulation of inositol phosphates could be due to their rapid degradation to inositol rather than lack of phospholipase C activation. Hallcher & Sherman (1980) have reported that lithium inhibits the phosphomonoesterase that degrades Ins*P*. The effect of Li^+ on the degradation of InsP_3 and InsP_2 has not been examined. Thus, as shown by Berridge *et al.* (1982) in their studies of agonist-dependent accumulation of the inositol phosphates in salivary glands, Li^+ can be used to prevent the breakdown of inositol phosphates to inositol and thus facilitate their detection.

To examine further the role of PtdIns(4,5)P_2 in the platelet response to thrombin, we have determined whether inositol phosphates accumulate in thrombin-stimulated, washed rabbit platelets in the absence and presence of Li^+.

Materials and methods

Materials

Thrombin (bovine), PtdIns(4,5)P_2 and PtdIns4*P* were purchased from Sigma. Phospholipid standards, except for PtdIns(4,5)P_2 and PtdIns4*P*, were from Serdary Research Laboratories (London, Ontario, Canada). Thin layer plates precoated with silica gel H (Baker Si250) were from Canlab (Toronto, Ontario, Canada). Solvents for t.l.c. were distilled before use.

[^{3}H]Inositol (15.8Ci/mmol) was from New England Nuclear and 5-hydroxy-[2-^{14}C]tryptamine creatinine sulphate (60mCi/mmol) was from Amersham/Searle Corp.

Preparation of prelabelled, washed rabbit platelets

Suspensions of washed platelets from three rabbits were prepared according to the method of Ardlie *et al.* (1971). The platelets were prelabelled in the first washing solution by incubation at 37°C for 1h with 20μCi of [^{3}H]inositol/ml of platelet suspension (2×10^9 platelets/ml). The unincorporated label was removed by centrifuging and resuspending the platelets twice in calcium-free Tyrode solution containing 0.35% albumin. Platelets were finally suspended at a concentration of 10^9/ml in Tyrode solution containing 0.35% albumin and apyrase at a concentration capable of converting 0.25μmol of ATP to AMP in 120s at 37°C. In experiments in which Li^+ was used, the Li^+ was included in a Tyrode/albumin solution in which the amount of Na^+ was decreased to maintain an osmolarity of 291mosmol/l. After resuspension in the Li^+-containing Tyrode/albumin medium, the platelets were incubated for 1 or 2h at 37°C. In 1h the Li^+ concentration in the platelets approaches 80% of the concentration in the medium and at 2h it is the same as the concentration in the medium (Imandt *et al.*, 1977).

To examine the release of amine granule contents from platelets stimulated with thrombin, the platelets were prepared as described above, except that they were labelled with [^{14}C]serotonin for 15min at the end of the 1h incubation in the first washing solution (Packham *et al.*, 1977). Release of [^{14}C]serotonin was determined by rapidly transferring the platelet sample from the aggregometer cuvette to a centrifuge tube 120s after the addition of thrombin and centrifuging at 8000*g* for 1.5min; radioactivity was then determined in a sample of the supernatant fluid.

Thrombin stimulation of prelabelled platelets

Platelets (0.5ml at 10^9/ml) prelabelled with [^{3}H]-inositol were treated with 0.1 or 1 unit of thrombin/ml. Aggregation was measured in a Payton Aggregation Module (Payton Associates, Scarborough, Ontario, Canada). The reaction was terminated at the specified times by addition of 250μl of ice-cold 30% (w/v) trichloroacetic acid with mixing. This mixture was transferred to a centrifuge tube and the aggregometer cuvette rinsed with an additional 250μl of 10% (w/v) trichloroacetic acid which was also transferred to the centrifuge tube. The acidified solution was cooled on ice for at least 10min and then centrifuged for

1min at 8000*g*. A portion of the supernatant (750μl) was removed and neutralized with NaOH in the presence of 0.5mM-Hepes buffer to stabilize the pH at 7.0. The solution was finally made up to 5ml by addition of water and frozen until chromatographed for isolation of the inositol phosphates.

Fractionation of inositol phosphates and determination of labelling

Aliquots of the platelet extracts (2.5ml) were fractionated on ion-exchange columns consisting of 2ml of BioRad AG1 X2 (200–400 mesh) ion exchanger in the formate form. The columns were eluted with the series of solvents described by Berridge *et al.* (1982); 20ml of each solvent was used and four fractions (5ml) were collected for each solvent directly into scintillation vials. After addition of 10ml of ACS counting cocktail (Amersham/Searle Corp.) the samples were mixed and the incorporated label counted in a Philips scintillation counter.

The identities of the fractions eluted from the columns were confirmed by preparing a mixture of ^{32}P-labelled InsP_3 and InsP_2 from red cell ghosts as described by Downes *et al.* (1982) and fractionating the mixture on the columns. Two well-separated peaks were obtained.

Determination of PtdIns(4,5)P_2 and PtdIns4P specific radioactivity

Platelets labelled with [^{3}H]inositol, as described in a previous section, were extracted consecutively with chloroform/methanol (1:2, v/v) and chloroform/methanol/2M-HCl (1:2:0.8, by vol.) to recover the inositol phospholipids as previously described (Vickers *et al.*, 1982). The lipids were fractionated by using a two-dimensional t.l.c. system and the spots containing PtdIns(4,5)P_2 and PtdIns4P scraped off and digested with $HClO_4$. Incorporation of [^{3}H]inositol and amount of inositol phospholipid, measured as inorganic phosphate, were determined as previously described (Vickers *et al.*, 1982).

Analysis of data

For all experiments, the prestimulation values were standardized to 10^9 platelets/ml and a total [^{3}H]inositol incorporation of 2×10^6d.p.m./10^9 platelets, which were approximately the average values. The factor to standardize the prestimulation values in each experiment was used to standardize the experimental results from that experiment. The standardized values were then averaged and the significance of the data was assessed by using a paired *t*-test.

Results

Effect of Li^+ on platelet aggregation and release

Incubation of platelets with 20mM-Li^+ for 1h resulted in a more rapid response of the platelets to 0.1 unit of thrombin/ml compared with platelets incubated in the absence of Li^+ (Fig. 1). Shape change had occurred and aggregation started 9s after thrombin addition in the platelets treated with 20mM-Li^+, compared with 12s in control platelets. Stimulation of platelets with 0.1 unit of thrombin/ml resulted in release of 63.0±1% of [^{14}C]serotonin at 120s in the absence of Li^+.

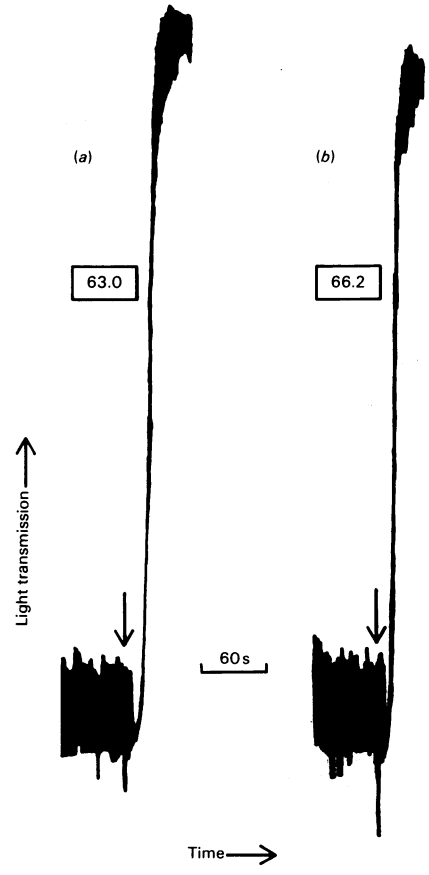

Fig. 1. *Tracings of light transmission through platelet suspensions stimulated with 0.1 unit of thrombin/ml of suspension in (a) the absence and (b) the presence of 20mM-Li^+*

The extent (%) of [^{14}C]serotonin release is shown in the boxes. Platelets were stirred for 1min prior to addition of thrombin indicated by the arrow. Samples were removed for determination of released [^{14}C]serotonin at 120s. The aggregation tracings are representative of the results of three experiments; the release data are the average of data from three experiments.

Li^+ (2.5–20mM) had little effect on the extent of release from platelets stimulated with 0.1 unit of thrombin/ml, the release in the presence of 20mM-Li^+ being $66.2 \pm 2.4\%$. Similar results were found in one experiment with platelets stimulated with 1 unit of thrombin/ml.

Effect of a range of concentrations of Li^+ on the accumulation of inositol phosphates

In platelets prelabelled with [^{3}H]inositol and stimulated with either concentration of thrombin (0.1 or 1.0 unit of thrombin/ml of suspension), Li^+ had little effect on the labelling of Ins$P$ at 30s (Fig. 2). At low concentrations of Li^+, the labelling of InsP_2 at 30s increased slightly while at higher concentrations (>5mM), it decreased to less than 50% of the value found in the absence of Li^+. In contrast, the labelling of InsP_3 at 30s showed little change below 5mM-Li^+, but with higher concentrations of Li^+ the labelling of InsP_3 increased to about 5 times the labelling found in the sample without Li^+. In subsequent experiments 20mM-Li^+ was used to inhibit the degradation of the inositol phosphates.

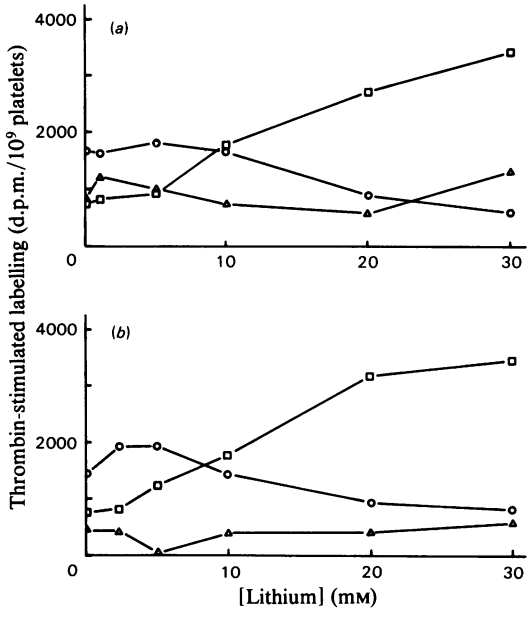

Fig. 2. *Changes in the [^{3}H]inositol labelling of the inositol phosphates due to thrombin stimulation of washed platelets in the presence of a range of Li^+ concentrations (0–30mM)*
In separate, single experiments platelets were stimulated with either (*a*) 0.1 or (*b*) 1.0 unit of thrombin/ml of suspension and the reaction terminated at 30s as described in the Materials and methods section. The inositol phosphates InsP_3 (□), InsP_2 (○) and Ins$P$ (△) were isolated by ion-exchange chromatography as described in the Materials and methods section. The results are from duplicate samples in one experiment for each of the thrombin concentrations.

Time course of inositol phosphate accumulation

In the absence of Li^+, the labelling of InsP_2 increased rapidly throughout the 120s period after the addition of thrombin to the platelets (Figs. 3*a* and 4*a*). The labelling of InsP_3 increased less rapidly and reached a plateau by 30s. The changes in the labelling of InsP_3 and InsP_2 in platelets

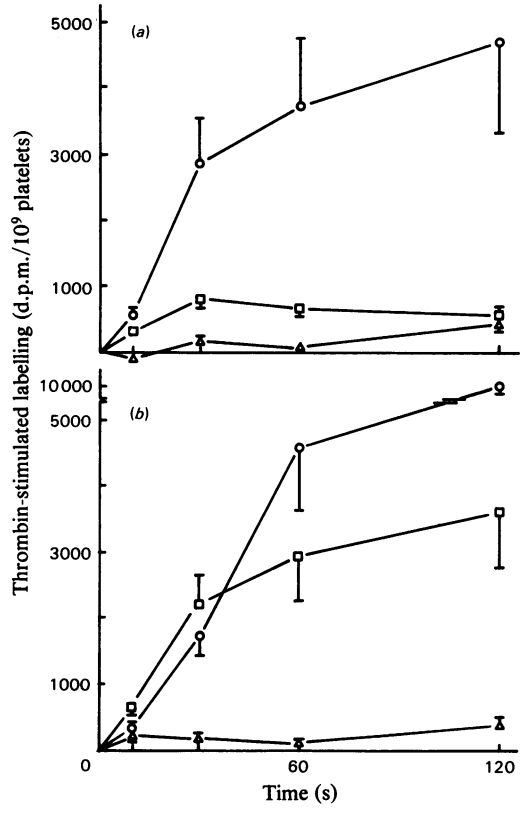

Fig. 3. *Time courses of the changes in labelling of InsP_3 (□), InsP_2 (○) and Ins$P$ (△) in response to stimulation of washed rabbit platelets prelabelled with [^{3}H]inositol with 0.1 unit of thrombin/ml of suspension in (a) the absence and (b) the presence of 20mM-Li^+*
The platelets were prepared, stimulated with thrombin and the inositol phosphates recovered as described in the Materials and methods section. In unstimulated platelets the labelling of the inositol phosphates was: InsP_3, 397, InsP_2, 613 and Ins$P$, 1764d.p.m./10^9 platelets. The total change in labelling of the inositol phosphates was 783d.p.m. in the absence of Li^+ and 1195d.p.m. in its presence at 10s and at 120s 5795d.p.m. in the absence of Li^+ and 14453d.p.m. in its presence. The results are the means of duplicate data from two experiments. Error bars represent S.E.M.

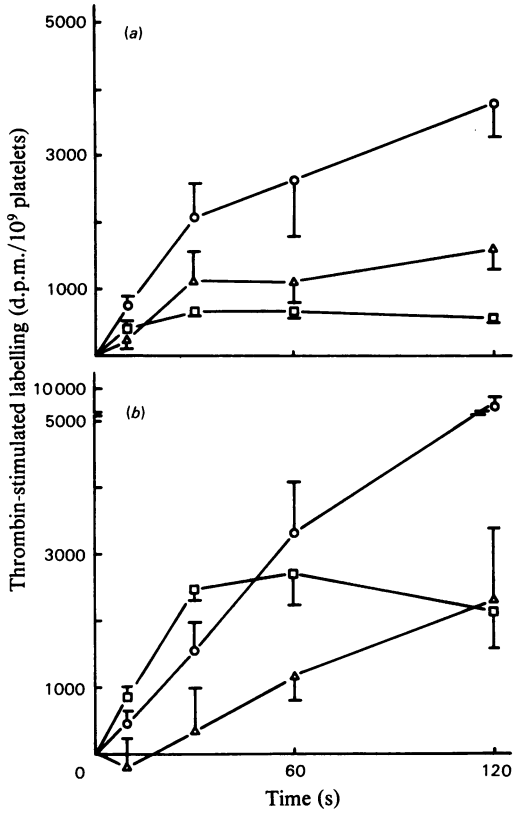

Fig. 4. *Time courses of the changes in labelling of InsP_3 (□), InsP_2 (○) and Ins$P$ (△) in response to stimulation of washed rabbit platelets prelabelled with [^3H]inositol with 1.0 unit of thrombin/ml of suspension in (a) the absence and (b) the presence of 20mM-Li^+*

In unstimulated platelets the labelling of the inositol phosphates was: InsP_3, 352, InsP_2, 428 and Ins$P$, 1010d.p.m./10^9 platelets. The total change in labelling of the inositol phosphates was 1346d.p.m. in the absence of Li^+ and 1105d.p.m. in its presence at 10s and at 120s 5986d.p.m. in the absence of Li^+ and 11789d.p.m. in its presence. Experimental design and data presentation are as described for Fig. 3.

stimulated with 0.1 or 1.0 unit of thrombin/ml were comparable. However, the change in Ins$P$ labelling, as a proportion of the total inositol labelling of the platelets, was much greater in platelets stimulated with 1.0 unit of thrombin/ml than with 0.1 unit of thrombin/ml (Fig. 4*a* versus Fig. 3*a*).

When the platelets had been preincubated with 20mM-Li^+ for 1 h, which is estimated to result in an internal concentration of about 15mM (Imandt *et al.*, 1977), the changes in the labelling of the inositol phosphates in response to thrombin were different from those found in the absence of Li^+. However, the changes in response to the two concentrations of thrombin were similar (Figs. 3*b* and 4*b*). In the presence of Li^+ the labelling of InsP_3 was increased and that of InsP_2 was decreased up to 30s in comparison with the results in the absence of Li^+. During the first 30s in the presence of Li^+, the labelling of InsP_3 was greater than the labelling of InsP_2. With the lower concentration of thrombin, the addition of Li^+ had little effect on the labelling of Ins$P$. With 1 unit of thrombin/ml, the labelling of Ins$P$ was suppressed in the first 30s, while by 120s the labelling of Ins$P$ was substantially greater than in the absence of Li^+.

Since the changes in labelling of the inositol phosphates could have been due to changes in specific radioactivity, the specific radioactivities of labelling of PtdIns4P and PtdIns(4,5)P_2 with [^3H]inositol were determined. Thrombin stimulation of platelets preincubated with Li^+ did not change the specific radioactivity at 60s of PtdIns(4,5)P_2 (2652±246d.p.m./nmol with thrombin compared with 2658±222d.p.m./nmol without thrombin) or PtdIns4P (2176±276 d.p.m./nmol with thrombin compared with 2022±275d.p.m./nmol without thrombin) in three experiments ($n = 6$).

Discussion

In the absence of Li^+, the major increase in the labelling of the inositol phosphates was in InsP_2. However, if the experiments were done in the presence of Li^+, which has been found to inhibit the phosphomonoesterase that degrades inositol 1-phosphate (Hallcher & Sherman, 1980), InsP_3 was labelled more rapidly than InsP_2. The observation that Li^+ decreased the rate of appearance of InsP_2 up to 30s and increased the rate of accumulation of InsP_3 is consistent with a substantial portion of the InsP_2, that is produced in the absence of Li^+ during this early period, resulting from the degradation of InsP_3 to InsP_2 by phosphomonoesterase. These results are compatible with the hypothesis that PtdIns(4,5)P_2 is the first phosphoinositide degraded in thrombin-stimulated platelets (Agranoff *et al.*, 1983; Billah & Lapetina, 1982; Imai *et al.*, 1983; Rendu *et al.*, 1983).

Agranoff *et al.* (1983) found that thrombin stimulation of human platelets led to a rapid increase in only InsP_3 with (in contrast to our observations) little subsequent change in this inositol phosphate or InsP_2. Marked differences in the experimental conditions between our experiments and those of Agranoff *et al.* (1983) may account for the differences in results. We have studied rabbit platelets suspended in a more physiological medium containing albumin and Ca^{2+}, whereas Agranoff *et al.* (1983) used suspen-

sions of washed human platelets in a medium which contained neither albumin nor Ca^{2+}. Furthermore it has been shown that Tris-buffered saline, which was used by Agranoff *et al.* (1983), produces changes in platelets that result in their response to stimuli differing from that of platelets in plasma or suspended in more physiological media (Packham *et al.*, 1984).

Ins*P* did not accumulate substantially in platelets stimulated with the lower concentration of thrombin in the absence of Li^+. This may indicate that Ins*P* was degraded as fast as it was produced by degradation of InsP_2 or PtdIns. However, Ins*P* also did not accumulate in the presence of Li^+. Thus either Li^+ did not block the degradation of Ins*P* or, alternatively, there was little degradation of PtdIns to Ins*P* under these conditions and Li^+ blocked degradation of InsP_2 to Ins*P*; the latter possibility would be consistent with the greater accumulation of radioactivity in InsP_2 in the presence of Li^+.

With the higher concentration of thrombin, the changes in InsP_2 and InsP_3 are comparable with those seen with the lower concentration of thrombin and are consistent with initial degradation of PtdIns(4,5)P_2 to 1,2-diacylglycerol and InsP_3; InsP_3 is then degraded to InsP_2. In this case Ins*P* accumulated in the absence of Li^+, indicating that its rate of production was greater than its rate of degradation. In the presence of Li^+ even more Ins*P* accumulated. This observation is consistent with the ability of Li^+ to block Ins*P* phosphomonoesterase. With this concentration of thrombin, the concentration of Li^+ used may not be sufficient to block completely the degradation of InsP_2 to Ins*P*. However, it is also possible that degradation of PtdIns to Ins*P* and 1,2-diacylglycerol may be activated by the higher concentration of thrombin. Whether the Ins*P* was produced by degradation of PtdIns or InsP_2 or both cannot be determined from these experiments. Increased degradation of PtdIns, which could reduce the amount of PtdIns for synthesis of PtdIns4*P* and PtdIns(4,5)P_2 and thus their degradation to InsP_2 and InsP_3, would be consistent with the observed smaller accumulation of radioactivity in InsP_3 and InsP_2 in platelets stimulated with the higher concentration of thrombin.

Previously Li^+ has only been reported to inhibit the phosphomonoesterase which degrades Ins*P* to inositol and phosphate (Hallcher & Sherman, 1980). The effect of the range of concentrations of Li^+ on the accumulation of the inositol phosphates implies that all of the phosphomonoesterases which degrade InsP_3 to inositol are inhibited by Li^+. The initial increase in InsP_2 labelling at low Li^+ concentrations (2.5–5mM), which is consistent with inhibition of InsP_2 degradation, and the increase in InsP_3 labelling only at higher concentrations, indicates that the phosphomonoesterases might have different sensitivities to Li^+.

There may be two mechanisms involved in increasing InsP_3 and InsP_2 in the absence and presence of Li^+. The initial increase in InsP_3 labelling was augmented by Li^+, indicating that InsP_3 produced in the absence of Li^+ was rapidly degraded to InsP_2. The plateau in InsP_3 labelling after 30s is consistent with Holmsen's hypothesis that only some changes are directly dependent on agonist–receptor interaction (Holmsen *et al.*, 1981). In the present experiments the receptor-dependent stimulation of the degradation of PtdIns(4,5)P_2 to 1,2-diacylglycerol and InsP_3 may be completed by 30s. Alternatively, a decrease in available PtdIns(4,5)P_2, due to degradation, may slow the rate of production of InsP_3. At this time shape change had occurred and aggregation was extensive. In other studies we have shown that by this time extensive release of the contents of the amine storage granules had occurred (Packham *et al.*, 1977). The slower increase in InsP_2 labelling, during the first 30s after stimulation in the presence of Li^+, indicates that degradation of PtdIns4*P* by phospholipase C, which would not be blocked by Li^+, was not strongly stimulated at early times. The increased accumulation of InsP_2 observed with Li^+ after 30s could be due to either increased degradation of PtdIns4*P* by phospholipase C or inhibition of breakdown of PtdIns4*P* to Ins*P* with a lesser inhibition of breakdown of InsP_3 to InsP_2. If later degradation of PtdIns4*P* by phospholipase C is involved, it may be mediated by an increase in cytoplasmic Ca^{2+} levels, since the phospholipases C involved are Ca^{2+}-dependent enzymes (Rittenhouse, 1983*b*).

Although the increases in inositol phosphate labelling observed in this study could be due to increases in specific radioactivity rather than increases in amount, this seems an unlikely explanation since the specific radioactivities of PtdIns(4,5)P_2 and PtdIns4*P* did not change in response to thrombin stimulation.

These experiments indicate that stimulation of platelets with thrombin causes an initial degradation of PtdIns(4,5)P_2 and a resultant increase in InsP_3, which is degraded to InsP_2. The degradation of PtdIns(4,5)P_2 may be directly activated by the interaction of thrombin with its receptor. Degradation of PtdIns4*P* may occur later, while degradation of PtdIns could be occurring in the presence of the higher concentration of thrombin. These degradation steps may be activated by some other mechanism; for example, an increase in cytoplasmic Ca^{2+}. Since we have previously observed that the amount of PtdIns(4,5)P_2 does not decrease with 0.1 unit of thrombin/ml (Vickers *et al.*, 1984)

and we have now found that InsP_3 accumulates, the results indicate that the production of InsP_3 and 1,2-diacylglycerol rather than the decrease in PtdIns(4,5)P_2 may be more important in the thrombin-induced platelet release reaction and aggregation.

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