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SEROTONERGIC AGONISTS STIMULATE INOSITOL LIPID METABOLISM IN RABBIT PLATELETS

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Summary

The metabolism of inositol phospholipids in response to serotonergic agonists was investigated in rabbit platelets. In platelets prelabelled with [^{3}H]-inositol, in a medium containing 10 mM LiCl which blocks the enzyme inositol-1-phosphatase, 5-hydroxytryptamine(5-HT)caused a dose-dependent accumulation of inositol phosphates (IP). This suggests a phospholipase-C-mediated breakdown of phosphoinositides. Ketanserin, a selective 5-HT$_2$ antagonist, was a potent inhibitor of the 5-HT response, with a Ki of 28 nM, indicating that 5-HT is activating receptors of the 5-HT$_2$ type in the platelet. Lysergic acid diethylamide (LSD) and quipazine also caused dose-related increases in inositol phosphate levels, though these were considerably less than those produced by 5-HT. These results show that relatively small changes in phosphoinositide metabolism induced by serotonergic agonists can be investigated in the rabbit platelet, and this cell may therefore be a useful model for the study of some 5-HT receptors.

5-Hydroxytryptamine (5-HT) has long been known to induce shape change and sometimes aggregation in platelets (1). Recently it has been found that activation of platelet 5-HT receptors elevates cytosolic [Ca^{2+}] (2) and increases protein kinase-C (PKC) activity (3). Mobilisation of intracellular [Ca^{2+}] and activation of PKC occur in response to other platelet activators (4,5,6), and it is thought that these responses are subsequent to receptor-mediated inositol lipid breakdown (7).

In the platelet rapid changes in the levels of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP$_2$) have been observed in response to various agonists, including thrombin (8,9), ADP (10) and platelet-activating factor (11). Recently it has been shown that phospholipase-C-mediated breakdown of the polyphosphoinositides to diacylglycerol (DG) and inositol polyphosphates is the initial event in thrombin- (12,13) and collagen- (14) stimulated platelets. Inositol trisphosphate (IP$_3$), derived from PIP$_2$, may mobilise intracellular [Ca^{2+}] stores (15,16) and DG is a potent activator of PKC (5).

Recently, a method using LiCl to block hydrolysis of

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inositol-1-phosphate (IP_1) to inositol has been developed (17) and this facilitates the measurement of small changes in phosphoinositide metabolism. This method has been employed to study the effects of serotonergic agonists on inositol lipid metabolism in rabbit platelets, which are permeable to inositol.

Methods

Blood was obtained from mature female New Zealand white rabbits (approximately 3 kg) by cardiac puncture under anaesthesia (pentobarbitone 30 mg/kg i.v.) and was taken into 3.15% trisodium citrate (1 vol:9 vol blood) plus 10 μM forskolin, which is a potent inhibitor of platelet aggregation (18). Platelet-rich plasma (PRP) was prepared by centrifugation at 160xg for 20min at 20°C. After a further addition of forskolin at the same concentration platelets were separated out by centrifugation at 800xg for 10min at 20°C. If the PRP was significantly contaminated with erythrocytes, an intermediate spin of 800xg for 5min was included prior to platelet sedimentation. Forskolin was then added prior to this spin and omitted from the final spin. Plasma was removed from the platelet pellet, which was then resuspended at a concentration of 0.5 ml for each 10 ml PRP in 5 mM Hepes/140 mM NaCl/4 mM KCl/1 mM MgCl/6 mM glucose/0.3% bovine serum albumin, pH 7.4. [^{3}H]-Myo-inositol was added at a concentration of 20 μCi/ml platelet suspension and the samples were incubated for 2h at 37°C with occasional mixing. The platelets were separated from extracellular label by elution off a Sepharose CL-2B column (19) with Hepes buffer in which NaCl had been replaced by equimolar choline chloride to prevent active uptake of 5-HT into the platelets (20), and glucose was increased to 10 mM. The column contained 8-10 ml gel for each 1 ml of platelet suspension and was equilibrated with 3-4 vol of choline-Hepes buffer prior to filtration of the platelets. After filtration the platelets were diluted in the choline-Hepes and LiCl was added at 10 mM final concentration (17). Aliquots (0.6 ml) were then taken into minivials in a shaking water-bath at 37°C and after 10min preincubation drugs were added as appropriate.

Incubations were stopped by the addition of 1.88 ml $CHCl_3/CH_3OH$ (1:2 v/v), followed by 0.62 ml $CHCl_3$ and 0.62 ml H_2O. Samples were then centrifuged at 1000xg for 5min and 1.5 ml of the upper phase was removed and diluted with 2.25 ml H_2O. Dowex 1-X8 (0.5 ml) (formate form, 100-200 mesh) was then added and the samples were mixed. Following three washes with 5 mM myo-inositol, inositol phosphates were eluted from the resin with 2 x 0.5 ml 1 M ammonium formate/0.1 M formic acid. After addition of 10 ml Instagel(Packard Corporation, Caversham, UK) the samples were counted. In some experiments inositol phosphates were separated on Dowex-formate columns as described by Godfrey and Putney (21).

[^{3}H]-Myo-inositol (specific activity 15 ci/mmol) was obtained from Amersham International, Amersham, UK; forskolin was obtained from Calbiochem-Behring, Hardwick, Cambridge, UK; Sepharose CL-2B, Dowex, choline chloride and Hepes were obtained from Sigma, Poole, UK; and all other reagents were of the highest grade available. Ketanserin was a gift from Janssen Pharmaceutical Limited, Grove, UK.

Results and Discussion

The addition of 30 μM 5-HT stimulated an accumulation of inositol phosphates (Fig. 1), a significant increase in IP levels was observed at 2min, and continued to increase throughout a 60min incubation. The levels of IP in unstimulated controls remained low during the experiment though a significant increase was observed during the 30-60min period (Fig. 1). For subsequent experiments the prelabelled cells were treated with agonist for 30min, thus providing an easily measurable response, without a substantial increase in unstimulated cells.

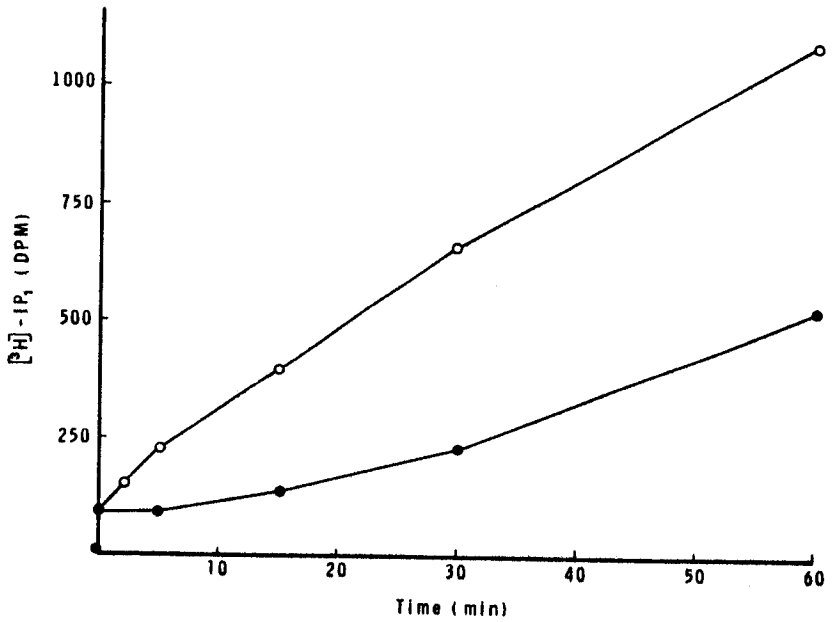

FIG. 1

Time course of inositol phosphate formation in cells stimulated with 5-HT. Cells were stimulated with 30 μM 5-HT (○), or vehicle alone (●) for the times indicated. Values were means of triplicates in a single experiment (SEM was less than 5% of the mean) and are corrected for all loss factors. Two further experiments gave similar results.

The increase in IP_1 was concentration-dependent over a 5-HT concentration range of 1-100 μM (Fig. 2). A maximal increase in IP formation was observed at 100 μM 5-HT and the EC_{50} was 8 μM (Fig. 2).

The addition of 100 nM ketanserin, a selective 5-HT_2 antagonist (22,23), and inhibitor of platelet 5-HT activation (2,3), moved the 5-HT dose-response curve to the right (Fig. 2). The parallel shift indicates that ketanserin is a competitive antagonist at this serotonin receptor. In the presence of higher concentrations of antagonist the response is almost totally blocked (Fig. 3); at 10 μM ketanserin inhibits the response to 100 μM 5-HT by over 80%. From these data the IC_{50} for ketanserin was observed to be 500 nM, and using the method of Cheng and Prusoff (24) the Ki was calculated to be 28 nM. This is comparable to a Kd of 18 nM obtained in [^{3}H]-LSD binding studies on platelet membranes (25) and provides further evidence that serotonin activates platelets via a 5-HT_2 receptor (26,27).

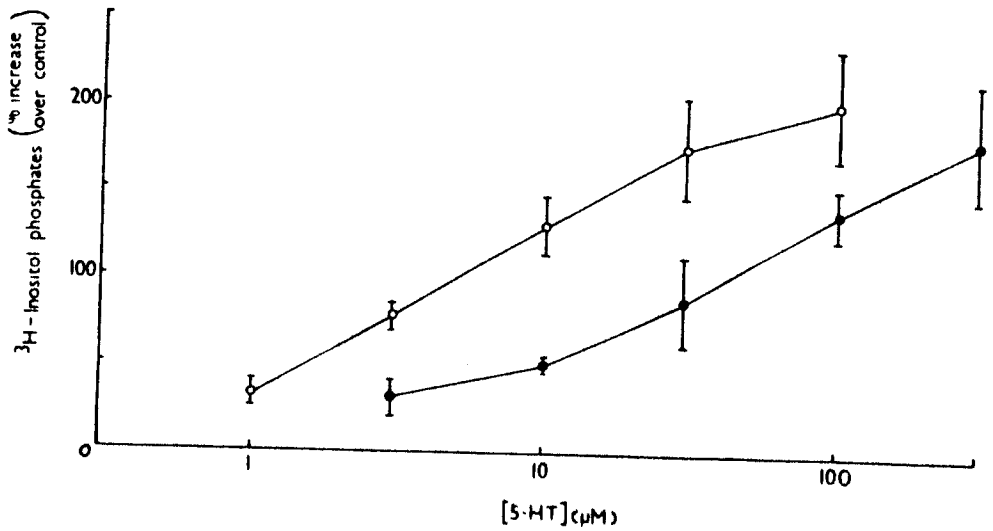

FIG. 2

Effects of 5-HT concentration on the formation of inositol phosphates. Cells were incubated with 5 HT for 30min. In experiments with ketanserin 100 nM ketanserin was added 10min prior to 5-HT. (o) 5-HT; (●) 5-HT + 100 nM ketanserin. Values are means ± SEM. (n = 4-5). The control values were (DPM); 5-HT 140 ± 23; + ketanserin 144 ± 33.

As breakdown of polyphosphoinositides is now thought to be the initial receptor mediated event (7), we attempted to measure changes in IP_2 and IP_3 levels. However, because 5-HT is only a weak agonist (1), we were unable to see any increase in the levels of inositol polyphosphates under our assay conditions

(results not shown).

Laubscher et al (28) showed that D-LSD induced shape change in rabbit platelets, although the magnitude of this response is much less than that to 5-HT. This study shows that LSD stimulated phosphoinositide turnover but, as might be expected, the maximal effect was considerably less than that produced by 5-HT (Fig. 4).

Quipazine is a 5-HT agonist which may be selective for 5-HT_2 receptors in the brain (29). This compound also increased IP_1 formation (Fig. 4). A maximal response was not reached at a concentration of 1 mM; higher concentrations could not be attained due to quipazine being relatively insoluble in an aqueous medium. Although it produced a greater increase in phosphoinositide turnover than LSD, its potency was considerably lower (EC_{50} for quipazine was >300 µM, whereas that of LSD was <100 nM).

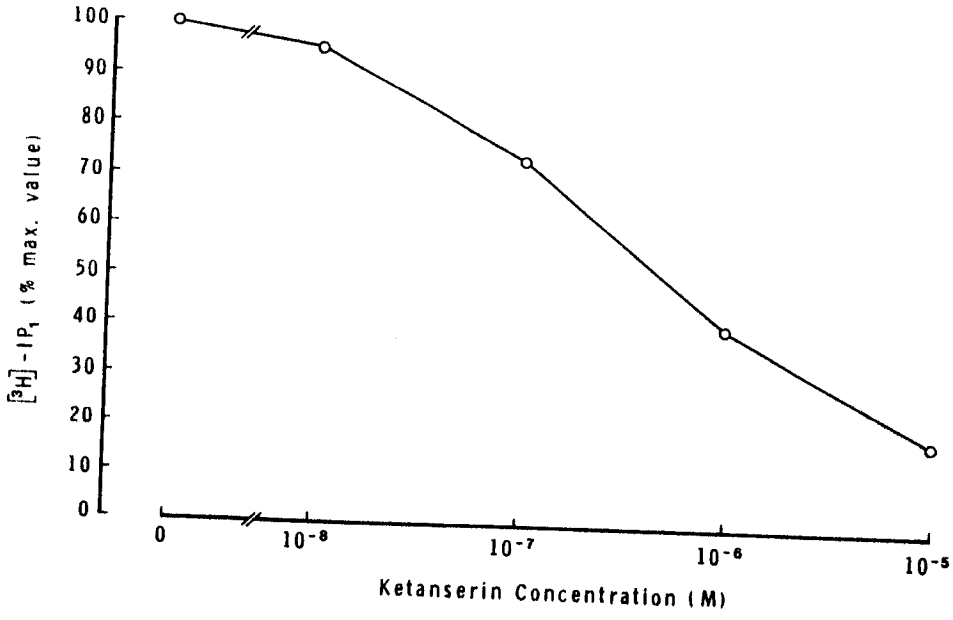

FIG. 3

Effects of increasing concentrations of ketanserin on the formation of inositol phosphates stimulated by 100 µM 5-HT. Ketanserin was added 10min prior to 5-HT and the platelets were stimulated for a further 30min. Results are means of triplicates in a single experiment (SEM was less than 10% of the mean), which was repeated twice with similar results. The 100% value was 328 ± 18 DPM.

Our findings are compatible with those of De Clerck et al (27) which suggested that 5-HT-induced platelet activation is mediated by receptors of the 5-HT_2 type (30). Ligand binding studies with [^{3}H]-LSD using human platelets (25) indicate that human platelet 5-HT receptors are also of this type and we have recently shown that rabbit platelet 5-HT receptors have similar characteristics (manuscript in preparation). This study also demonstrates that Li^+ can be used to amplify relatively small changes in platelet phosphoinositide metabolism, such as those produced by 5-HT and other serotonergic agonists. This extends the usefulness of the platelet as a model for some 5-HT receptors since ligand binding studies can be complemented by measurements of a defined biochemical response.

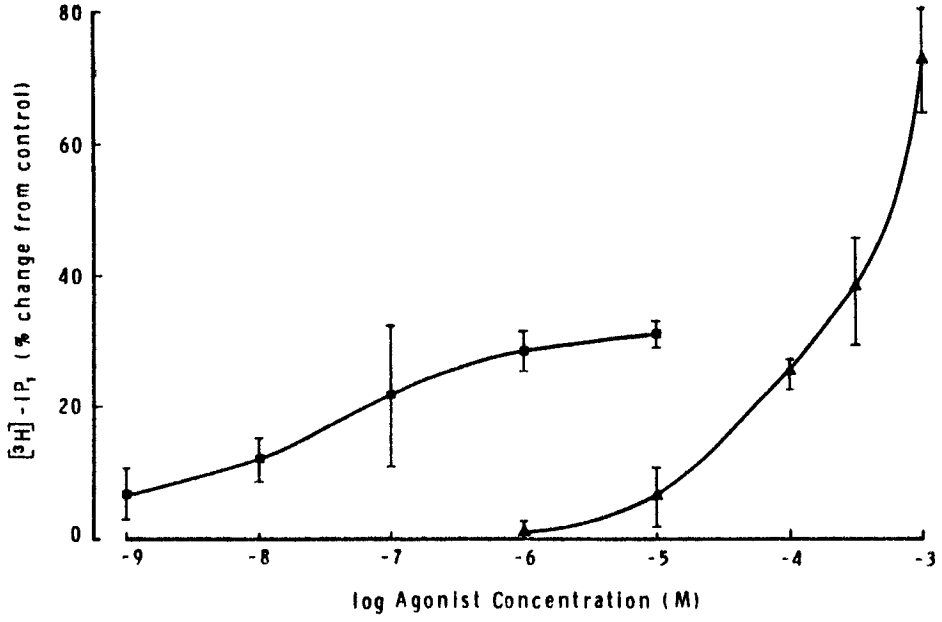

FIG. 4

Effects of LSD and quipazine concentration on formation of inositol phosphates. Platelets were incubated with agonist for 30min. Values are means ± SEM (n = 5, LSD; n = 4, quipazine). Control values were (DPM); LSD, 239 ± 107; quipazine, 304 ± 106.

Acknowledgements

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