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Localisation of the [^{32}P]IP_3 binding site on human platelet intracellular membranes isolated by high-voltage free-flow electrophoresis

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This study reports the localisation of the [^{32}P]IP_3 binding site on highly purified membrane fractions prepared using high-voltage free-flow electrophoresis. Binding studies on mixed membranes, carried out at 4°C, revealed a binding site with a K_d = 86 nM and β_{max} = 5.3 pmol/mg protein. The binding was potently inhibited by heparin. High-voltage free-flow electrophoresis was used to further purify surface and intracellular membranes. The intracellular membranes showed a 5-fold enrichment of binding sites with respect to the parent mixed membranes with the same K_d (80 nM), but the surface membranes showed an absence of binding activity. The results indicate the localisation of the IP_3 receptor on highly purified intracellular membranes.

Inositol trisphosphate; Binding site localisation; Human platelets

1. INTRODUCTION

The activation of platelets by agents such as thrombin and thromboxane leads to the formation of inositol phosphates and the elevation of cytosolic Ca^{2+} levels. The role of inositol trisphosphate (IP_3) in causing Ca^{2+} release from intracellular stores is well established [1], however the contribution played by IP_3 in Ca^{2+} influx is still controversial. Whether the effect on Ca^{2+} influx occurs as a direct interaction with the plasma membrane or indirectly via an interaction with the intracellular stores remains to be resolved. The localisation of binding sites on highly purified membrane fractions may help resolve this controversy. Binding sites on particulate fractions for radiolabelled IP_3 have been characterized in many different tissues (e.g. [2–6]) including platelets [7,8] with dissociation constants varying from 0.1–70 nM depending upon the tissue, method of preparation of membranes and the conditions of the binding assay. Additionally, the IP_3 receptor has been purified from a number of tissues [9,10] and cloned [13]. The purified receptor when reconstituted into liposomes has also been shown to mediate Ca^{2+} flux [14] and single channel activity [15].

In all of the binding studies reported thus far the particulate preparations are impure and contain elements from both plasma and intracellular membranes and thus a localization of the receptors for IP_3 on the endoplasmic reticulum (ER) where its Ca^{2+} releasing action has been established, has not been exclusive. Indeed, a number of binding studies have revealed the site to be on membrane structures that co-migrate with plasma membranes [16–18] and even in nuclei [19]. IP_3 has also been suggested to have direct actions on plasma membrane channels in T-lymphocytes [20] and also on Ca^{2+} fluxes on plasma membrane fractions prepared using Percoll density gradients from platelets [21]. Immunohistochemical studies on Purkinje cells using antibodies have provided clearer evidence that the IP_3 receptor is distributed on all regions of the ER and the nuclear envelope [22,23] but particularly on the smooth ER [24].

The purpose of this study was to determine the localisation of the [^{32}P]IP_3 binding site using highly purified preparations of platelet surface and intracellular membranes prepared by high-voltage free-flow electrophoresis. Previous studies have extensively characterized these membrane fractions with respect to protein and lipid constituents, marker enzymes [25,26], the ability of intracellular membranes to sequester Ca^{2+} [27,28] and to release it with IP_3 [29]. This study reports on the localisation of the [^{32}P]IP_3 binding site on these membrane systems.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]Inositol trisphosphate (1000 Ci/mmol) and unlabelled inositol trisphosphate were obtained from Amersham International (UK). Neuraminidase (type X) was purchased from Sigma Chemical Co., Dorset, UK. All other reagents were of analytical grade.

2.2. *Methods*

The procedure for the preparation of surface and intracellular membranes, i.e. separation of platelets from blood, neuraminidase treatment, sonication of platelets, separation of mixed membranes on sorbitol density gradients and further fractionation into intracellular and surface membranes by high-voltage free-flow electrophoresis using a VAP 5 apparatus was identical to that described in [29] with the following modifications. During sonication of the platelets the protease inhibitor cocktail contained 0.3 U/ml aprotinin, 5 μg/ml pep-

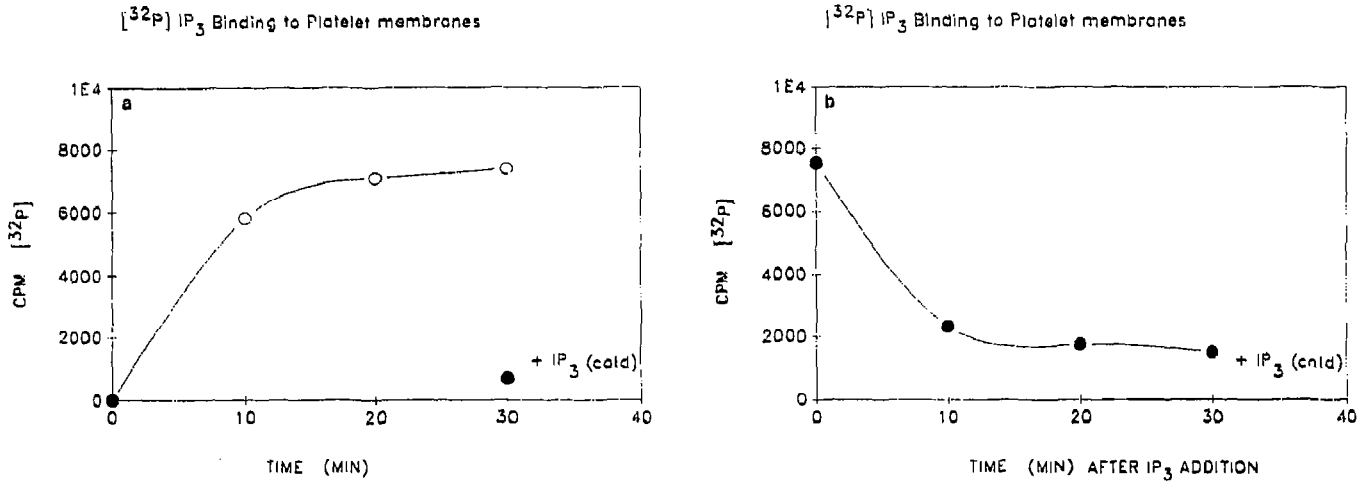

Fig. 1. Kinetics of [^{32}P]IP_3 binding to platelet mixed membranes and its displacement. (a) Incubations containing 0.1 nM [^{32}P]IP_3 are carried out as described in section 2 and reactions stopped by rapid filtration. (b) Unlabelled IP_3 (1 μM) is added after 20 min incubation. Total cpm in the reactions represent 33802 cpm. Points are means of duplicates with three other preparations showing similar results.

statin A and 200 μM PMSF. At the end, the membrane fractions were concentrated by centrifugation at 100,000 × g for 1 h and resuspended in 0.34 M sorbitol, 10 mM HEPES pH 7.2 for analysis.

Binding of [^{32}P]IP_3 to the membrane fractions was carried out using a modification of the procedure by Worley et al. [3] and Tones et al. [30]. Reaction mixtures in 0.5 ml contained 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 25 mM Na_2HPO_4, 1 mg/ml BSA, pH 8.0, 0.1 nM [^{32}P]IP_3, 200 μg membrane protein (or 100 μg of intracellular membranes) and where stated varying concentrations of unlabelled IP_3. Incubations were carried out at 4°C for 30 min after which 450 μl aliquots were rapidly filtered through a 0.45 μ filter, which was then washed twice with 5 ml of a buffer containing 0.25 M sorbitol, 10 mM K_2HPO_4 pH 7.0. The radioactivity on the filter was counted by liquid scintillation.

3. RESULTS AND DISCUSSION

The binding of [^{32}P]IP_3 was initially determined on mixed membranes prepared from platelets using sorbitol density gradients. Fig. 1a shows the time course of the binding of 0.1 nM [^{32}P]IP_3 to platelet membranes at 4°C. Binding is rapid over the first 10 min and reached equilibrium thereafter. Addition of 1 μM non-labelled IP_3 at equilibrium (after 20 min incubation) leads to a displacement of [^{32}P]IP_3 such that less than 10% is remaining after 30 min incubation (Fig. 1b). Inhibition of [^{32}P]IP_3 binding also occurs to the same extent if unlabelled IP_3 (1 μM) is added at the start of the incubations. The displacement of [^{32}P]IP_3 by unlabelled IP_3 were further characterised using different concentrations of unlabelled IP_3 (1–1000 nM) and Fig. 2a shows a typical displacement curve with concentrations of 500 nM IP_3 and above giving maximal displacement. Analysis of the data using a Scatchard plot (Fig. 2b) gave a straight line by regression analysis with a K_d = 86 nM and a binding capacity (β_{max} = 5.3 pmol/mg protein).

The binding site was then further characterized examining the effects of various agents added at the start of the incubations. Inositol 1,4-bisphosphate shows only very minimal displacement at high concentrations (up to 50 μM showing no effect and 100 μM giving 38% displacement). Heparin has been shown in many studies to antagonise the actions of IP_3 and to displace IP_3 from

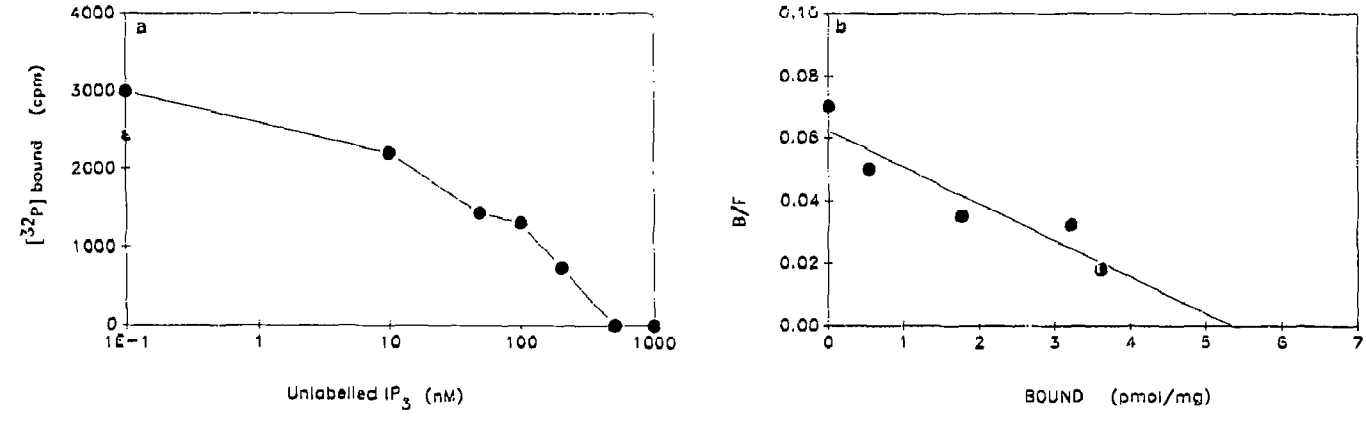

Fig. 2. Displacement (a) and Scatchard analysis (b) of [^{32}P]IP_3 binding to platelet mixed membranes. Incubation conditions as in section 2. Points are means of duplicates and the data shown is typical of three membrane preparations.

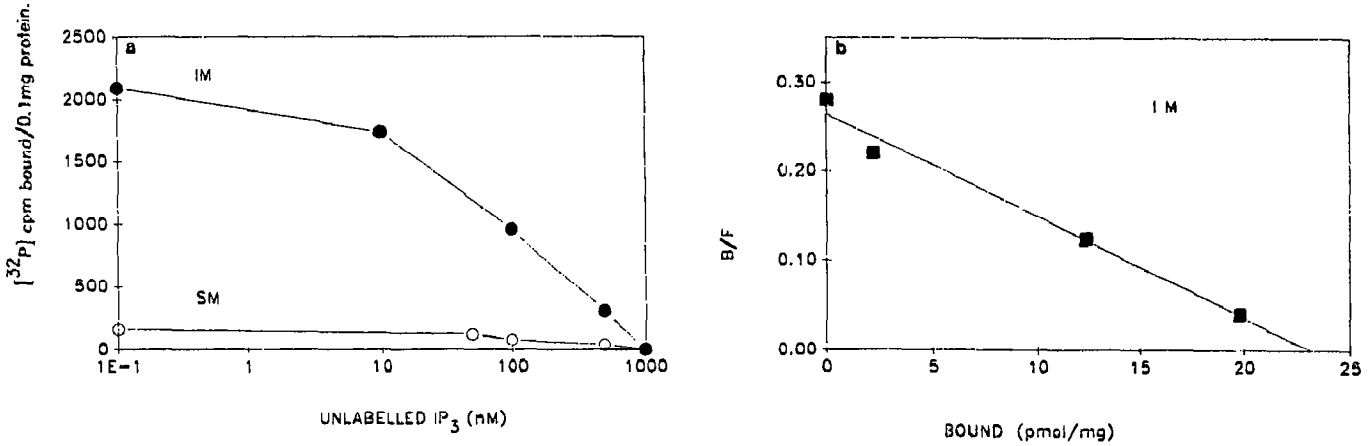

Fig. 3. Binding and displacement of [^{32}P]IP_3 binding to purified surface and intracellular membranes. (a) 100 μg of membrane fractions were incubated with 0.1 nM [^{32}P]IP_3 and varying concentrations of unlabelled IP_3. Total radioactivity in reactions represent 38037 cpm. IM, intracellular membranes; SM, surface membranes. (b) Scatchard analysis of the data for intracellular membranes. Due to small amounts of purified membranes obtained points are single determinations with two other preparations showing similar results.

its binding site. Using mixed membranes, unfractionated heparin potently displaced [^{32}P]IP_3 from its binding site with an EC_{50} = 2 μg/ml and total displacement shown at concentrations greater than 10 μg/ml (results not shown).

Studies were then carried out to localise the IP_3 binding, using membranes purified by free flow electrophoresis to yield surface and intracellular membranes. As indicated earlier these membrane fractions have been extensively characterized [25,26]. The surface membrane fractions are particularly rich in surface glycoproteins and do not sequester Ca^{2+} via an ATP-dependent mechanism and thus an effect of IP_3 on Ca^{2+} fluxes across the purified plasma membranes cannot be directly tested [27,28]. The intracellular membranes however have been shown to sequester Ca^{2+} and have the associated Ca^{2+}, Mg^{2+} ATPase activity [27,28]. Fig. 3a shows the binding of [^{32}P]IP_3 to the purified membrane fractions and its displacement by various levels of unlabelled IP_3. The intracellular membranes exhibited binding of [^{32}P]IP_3 which was displaced by increasing concentrations of unlabelled IP_3. Scatchard analysis revealed a K_d = 80 nM with an increased binding capacity of 23 pmol/mg protein (Fig. 3b). The surface membranes however showed little binding with some preparations showing no difference between specific and non-specific binding. Fig. 4 shows analysis of data from 3 different membrane preparations comparing binding activities of the purified fractions with respect to the parent mixed membranes. There is a clear enhancement of binding activity (5-fold) in intracellular membranes with a marked absence of this activity in surface membranes.

As indicated earlier, the role of IP_3 in releasing Ca^{2+} from intracellular stores is well established. This study shows that using highly purified platelet intracellular and surface membranes that the [^{32}P]IP_3 binding site is localised exclusively on intracellular membranes in line with its ability to release Ca^{2+} from these stores. A number of studies have suggested the direct involvement of IP_3 in ion fluxes across the plasma membrane, e.g. in T-lymphocytes [20] and in plasma membranes prepared from platelets using Percoll density gradients where IP_3 was reported to induce Ca^{2+} flux in membranes loaded with Ca^{2+} via a Na^+/Ca^{2+} exchange mechanism [21]. However in the latter study it is not clear whether this effect is due to a specific binding site on the membranes or a non-specific effect due to the abnormal dose–response relationship that was reported. The absence of a measurable binding site for IP_3 in platelet plasma membranes prepared using free flow electrophoresis provides evidence against a direct role for IP_3 in

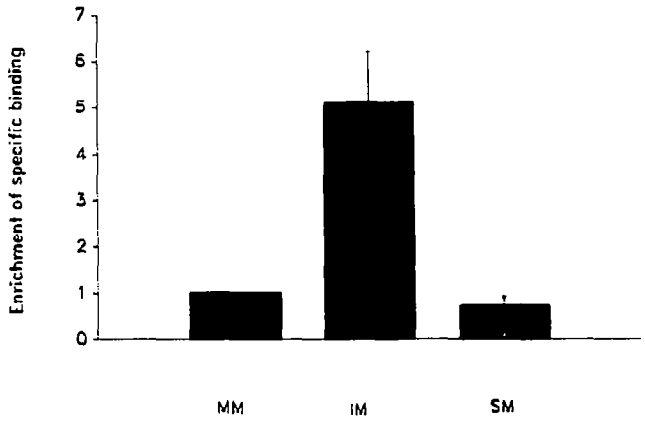

Fig. 4. Comparison of [^{32}P]IP_3 binding to intracellular and surface membranes with respect to the parent mixed membranes. The data are calculated from the specific binding of 0.1 nM [^{32}P]IP_3 to membrane fractions and then expressed as fold enrichment with respect to the activity in the parent mixed membranes (which ranged between 10–16 fmol IP_3 bound/mg protein). MM, mixed membranes; IM, intracellular membranes; SM, surface membranes. The data represent means ± S.E.M. of three determinations on different membrane preparations.

mediating Ca^{2+} fluxes across the plasma membranes. The IP_3 sensitive Ca^{2+} store has been suggested to be a specialised organelle which may either be a part of, or distinct from the endoplasmic reticulum [31,32], and it probably contains the Ca^{2+} binding protein calreticulin in the lumen [33–35]. Whether the platelet intracellular membranes prepared by free flow electrophoresis contain calreticulin remains to be determined.

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