Volume 254, number 1,2, 52–58 FEB 07527 August 1989

High incorporation of [^{3}H]inositol into phosphoinositides of human platelets during reversible electropermeabilisation

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Received 3 July 1989

A new method for high incorporation of [^{3}H]inositol into human platelets is described. The method involves incorporation of [^{3}H]inositol during reversible electropermeabilisation by high voltage discharge, followed by resealing the cells during incubation at 37°C. Between 10- and 20-fold increase of isotope uptake is achieved compared to control intact cells. Permeabilised resealed platelets maintain good responses to thrombin and collagen. Analysis of the incorporation of the label amongst the phosphoinositides shows 70% to be in PI, 20% in PIP, and 10% in PIP_2. Stimulation with thrombin and analysis of the formation of IP_1, IP_2 and IP_3 shows the labelling to occur in a hormone-sensitive pool. These studies indicate that reversible electropermeabilisation can be used to achieve good uptake of non-membrane penetrating substances such as inositol.

Inositol uptake, [^{3}H]; Reversible electropermeabilisation, (Human platelet)

1. INTRODUCTION

Over the last few years the link between receptor activation, phosphoinositide metabolism and intracellular Ca^{2+} mobilisation in cells and tissues, has been the focus of considerable research interest. In blood platelets such intracellular biochemical events are also associated with the action of certain agonists as part of their normal haemostatic functions. Although other transmembrane signalling mechanisms also exist in platelets it is now well established that the action of agonists such as thrombin, platelet activating factor (PAF), thromboxane A_2, PGH_2 and serotonin can all result in the degradation of phosphoinositides and the formation of the second messengers IP_3 (inositol 1,4,5-trisphosphate) and 1,2-diacylglycerol [1–4]. The former metabolite is involved in regulating Ca^{2+} movement out of intracellular storage compartments [5] and the diacylglycerol is associated with the action of protein kinase-C in important phosphorylation mechanisms [6]. Such findings have led to an interest in radioactively labelling the platelet phosphoinositide pool so as to follow routes of metabolism and synthesis during agonist-induced activation. A major difficulty experimentors face, however, in the planning of dynamic studies of the phosphoinositide pools, is that one of the most appropriate labels [^{3}H]inositol penetrates the platelet plasma membrane very poorly and the rate and extent of its incorporation into phosphoinositides are extremely low. Although alterations to the incubation medium (e.g. lowering the glucose concentration and addition of Mn^{2+}) have been reported to enhance the level of incorporation, generally the increases observed have been rather modest [7,8]. Additionally with platelets, long incubation times, which can carry the risk of surface and intracellular protease attack, may be necessary.

Recently we have reported the construction of suitable equipment and the establishment of optimum conditions for reversibly electropermeabilising the plasma membrane of human and animal

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

platelets using cycles of high-voltage discharge [9]. With optimum voltage conditions and the appropriate number of high-voltage discharge cycles, the membrane can be rendered permeable to small molecular mass substances (nucleotides, fluorescent probes, inositol phosphates etc.) and resealed without loss of morphological and functional integrity. Greater than 86% of rat platelets and close to 100% of human platelets became permeable during the high-voltage discharge and reseal after a period of incubation at 37°C. Full receptor status for activating ligands is well maintained and, if ATP is included in the medium during the permeabilisation, aggregation and secretion responses differ little from those seen with control non-permeabilised platelets. Moreover, in studies with rat platelets, the reinfusion into the circulation of permeabilised and resealed platelets labelled with ^{111}Indium results in essentially normal life span characteristics [9].

In this paper we describe the use of this permeabilising/resealing protocol to enhance the incorporation of [^{3}H]inositol into the human platelet phosphoinositide pools. Between 10- and 20-fold increases in [^{3}H]inositol uptake can be achieved when compared with non-permeabilised control cells. The time course of incorporation can be reduced, thus avoiding proteolytic artefacts and importantly, reasonable radioactive counts can be obtained for metabolites at substantially lower external concentrations of the label. Experimental costs are thus substantially less than with intact cell labelling.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All reagents were of analytical grade wherever possible and solutions prepared in either bidistilled water or analytical grade water (BDH, Poole, Dorset). The nucleotides ATP, CTP and ITP were obtained from Sigma, Poole, Dorset, as also were thrombin and the phospholipid standards PI, PIP and PIP_2. The [^{3}H]inositol (110 Ci/mmol) and [^{3}H]IP_3 (1.5 Ci/mmol) were purchased from Amersham International, UK. The apparatus used for electropermeabilisation was based upon a design reported by Knight and Scrutton [10] and constructed by Rank Bros Ltd, of Bottisham, Cambridge, England. Details of this equipment and the discharge chamber have been reported in an earlier communication [9]. The discharge chamber for platelets has a capacity of $\approx 900\ \mu l$ and the gap between the electrodes is 3 mm. The capacitor discharge was estimated to decay exponentially with a half-life of about 13 μs under the conditions suitable for platelet permeabilisation and temperature changes within the chamber were negligible.

2.2. *Preparation of platelet suspensions and electropermeabilisation*

All platelet preparations were made from citrate anticoagulated blood as reported in detail in earlier publications [9,11]. In some experiments the platelets were prelabelled with 5-hydroxy[^{14}C]tryptamine and this was carried out at the platelet-rich plasma (PRP) stage before isolation and washing. After separation from the PRP and resuspension and acidification to pH 6.5 with citric acid they were centrifuged ($1200 \times g$ for 10 min) and resuspended in the 'permeabilising buffer' which consisted of 150 mM potassium glutamate, 20 mM Pipes, 7 mM $MgCl_2$, 5 mM potassium ATP, 5 mM glucose and 1 mM EGTA. This buffer was adjusted to pH 7.4 and 100 nM PGE_1 was included before suspending the cells. After a period of equilibration in this buffer (≈ 45 min at room temperature) aliquots of the platelet suspension ($5–8 \times 10^8$ cells/ml) were carefully transferred to the discharge chamber avoiding bubble formation. In some experiments CTP or ITP (5 mM) were substituted for the ATP in the buffer and if all nucleotides were omitted the $MgCl_2$ concentration was reduced to 2 mM. For the electropermeabilisation generally 7 cycles of high-voltage discharge were used applied at 30–60 s intervals at the stated discharge voltages (see results). The efficacy of the permeabilisation and resealing, which occurs when the suspension is incubated at 37°C (30–60 min), was monitored by including Lucifer yellow (500 μg/ml) in some suspensions and examination of the cells by fluorescent microscopy, as earlier described. More routinely the permeabilisation and resealing can be monitored by an apparent upward platelet volume shift when aliquots of the cell suspension (transferred to ISOTON II) are immediately volume analysed using a resistive particle counter (Coulter ZM with C256 Channelyser). In experiments involving [^{3}H]inositol incorporation the label was included in the permeabilisation buffer before application of the discharge cycles. Resealed platelets were allowed to return to room temperature, acidified to pH 6.5 with citric acid, centrifuged ($500 \times g$ 15 min) and then resuspended at a concentration of 3×10^8 cells/ml in a Ca^{2+}-free tyrode medium consisting of 135 mM NaCl, 4 mM KCl, 6 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 1 mM glucose, 10 mM Hepes, 1 mM $MgCl_2$, pH 7.4.

Aggregation studies were carried out with 0.5 ml aliquots of platelet suspension using a Payton 300B dual channel aggregometer. Secretion studies with platelets prelabelled with [^{14}C]5HT were performed exactly as described in earlier reports [9,11].

2.3. *Analysis of [^{3}H]phosphoinositides [^{3}H]inositol phosphates*

The [^{3}H]inositol-labelled lipids were extracted from 3×10^8 platelets using the procedure described by Billah and Lapetina [12] with a solvent consisting of chloroform/methanol/conc. HCl (20:40:1, v/v). The chloroform extracts were applied to TLC plates pre-impregnated with 1% potassium oxalate in 10 mM EDTA, pH 6.5. Separation of the different phosphoinositides was carried out with the solvent system chloroform/methanol/4 M NH_4OH (9:7:2, v/v) with standards to assist location. Bands were visualised using iodine vapour and the areas corresponding to PI, PIP and PIP_2 scraped off and the radioactivity counted by liquid scintillation counting. Analysis

of the formation of inositol phosphates was carried out exactly using the method of Downes and Wusteman [13] using Dowex-formate columns. Platelet suspensions (0.5 ml) were treated with thrombin (1 U/ml) for 15, 30 and 45 s and stopped with 400 μl ice-cold 15% TCA. The mixtures were then extracted with ether, neutralised with 5 mM disodium tetraborate, applied to 2.0 × 0.5 cm Dowex-formate columns and the inositol phosphates eluted with increasing concentrations of ammonium formate. The eluates were collected in 2 ml fractions and from each a 250 μl aliquot was taken for scintillation counting. All fractions containing concentrations of ammonium formate greater than 0.8 M required the addition of 1 ml of water to the scintillant mixture to overcome quenching effects. The eluted peaks were identified using [^{3}H]inositol phosphate standards.

3. RESULTS

Earlier studies have shown that human platelets exposed to 7 discharge cycles at field strengths between 6 and 8 kV/cm become freely permeable to small molecular mass substances (limit 600 Da). Incubation at 37°C for 30–60 min results in resealing, the plasma membrane integrity is restored and this permeability is lost. Studies with non-membrane penetrating fluorescent probes have demonstrated that after resealing >85% of rat platelets and close to 100% of human platelets contained the encapsulated fluorochrome [9]. Routinely the efficacy of electropermeabilisation was followed by Coulter Counter volume analysis. Permeabilisation of the platelet membrane in the high K^+ medium is associated with an upward shift in 'apparent modal volumes' from 6–7 fl to as high as 12–14 fl when the platelets are transferred to ISOTON II for size analyses. After 60 min incubation in the high K^+ medium after electrical discharge treatment and then transfer to ISOTON II, the normal size distribution profile is restored [9].

Fig.1 shows that the inclusion of 8 μCi/ml [^{3}H]-inositol in the surrounding buffer medium during the high-voltage discharge cycles (7 cycles at 7 kV/cm) results in a maximum uptake of the label occurring around 60 min at which time the intracellular content of the label is at least 15–20 times higher than can be achieved with non-permeabilised control platelets exposed to the same concentration of [^{3}H]inositol. That the uptake plateau is in full accord with the studies of the resealing time course using fluorescent probes, and other radiolabelled agents, as also with the restoration of normal volume profiles in the Coulter size analyser. Similarly, Ca^{2+}-induced granule 5HT

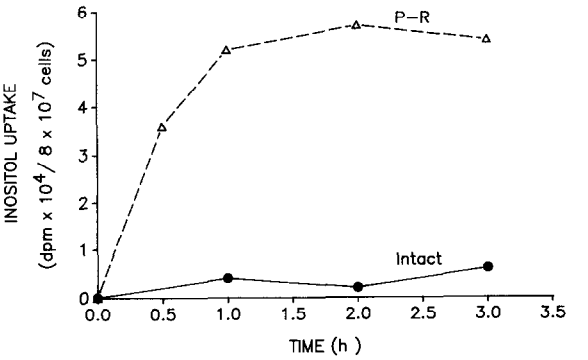

Fig.1. Time course of [^{3}H]inositol incorporation into permeabilised resealed human platelets. Cells were resuspended at 8 × 10^8 cells/ml and electropermeabilised at 7 kV/cm (7 discharges) (Δ) in the presence of 8 μCi/ml [^{3}H]inositol, before incubation at 37°C for various times. Aliquots (100 μl) were removed and placed in 0.4 μM filters and washed rapidly with 3 × 5 ml of cold buffer. The filters were counted by liquid scintillation. Intact cells (●) were treated identically except that no permeabilisation was carried out.

secretion and IP_3 stimulated release of intracellularly sequestered Ca^{2+}, two properties well expressed immediately after high-voltage discharge, are no longer demonstrable after the incubation period for resealing [9]. In the series of investigations reported here the uptake of [^{3}H]inositol into non-permeabilised control platelets amounted to 0.34% ± 0.11 (n = 4) of the available external label and with five different platelet suspensions permeabilised and resealed at 7 kV/cm in the presence of 8 μCi/ml [^{3}H]inositol, the mean uptake into the cells was 3.6% ± 1.9 (n = 5) of the available label. Studies were also carried out to establish the optimim high-voltage discharge conditions for this inositol uptake and for the maintenance of morphological and functional integrity after resealing with respect to receptor status for the activators thrombin and collagen. Fig.2 shows that with 7 discharge cycles, at field strengths between 7 and 14 kV/cm, the uptake of ^{3}H increases with increasing field strengths and, at 11 kV/cm, the % within the cells of available label was >20-fold higher than for control platelets from the same donor not exposed to the permeabilisation/resealing protocol. With respect to agonist induced aggregation responses, at field strengths of 7 and 9 kV/cm, thrombin (0.2 U/ml) gave good activation responses with only small decreases in % aggregation (90 and 86% of con-

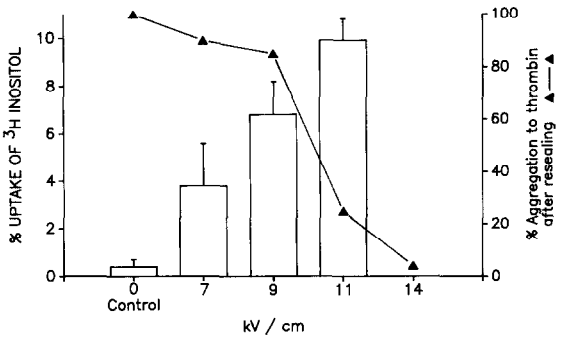

Fig.2. The effects of different voltages during electropermeabilisation on [^{3}H]inositol uptake and activation responses to thrombin in permeabilised resealed platelets. All incubations contained 8 μCi/ml [^{3}H]inositol in the permeabilisation buffer. Platelet suspensions were electropermeabilised with 7 discharges at different voltages and then incubated for 2 h at 37°C. The cells were then centrifuged and resuspended before estimations of [^{3}H]inositol uptake and aggregation responses (extents) to 0.2 U/ml thrombin were made. Results are expressed as % uptake (± SD) of ^{3}H label recovered in cells with respect to total added in the original incubation. Percentage uptake for [^{3}H]inositol for 14 kV/cm was not determined. Control cells (0 kV) were treated identically except no permeabilisation was performed. Extent of aggregation to thrombin is expressed as a percentage of that obtained with control non-permeabilised platelets.

trols respectively), as did collagen also (10 μg/ml, results not shown). The aggregation response after 7 cycles at 11 kV/cm were however substantially reduced (to around 25% of controls) and at 14 kV/cm the response to thrombin was effectively lost (<5% of controls). Previous studies have shown that with 7 discharge cycles at 14 kV/cm followed by an incubation period, the addition of Ca^{2+} at 10^{-5} M to the external medium in the presence of 5 mM ATP resulted in a 40% release of granule stored 5-hydroxy[^{14}C]tryptamine [9]. It therefore appears that under these higher discharge conditions the membrane remains permeable, allows the influx of Ca^{2+} and granule membranes can fuse to the plasma membrane for exocytosis. The receptor status for surface activating ligands may be compromised or some features of the signal transduction mechanisms affected at discharge levels >11 kV/cm. Because intracellular adenine nucleotides are lost during permeabilisation, all permeabilisation and resealing studies here have been carried out in a medium containing 5 mM ATP. In the lipid cycle concerned with the formation of phosphatidylinositol, CTP is a vital intermediate. However, substitution of either CTP or ITP (at 5 mM) for the ATP in the surrounding medium during electropermeabilisation (8.5 kV/cm, 7 cycles) gave essentially the same values as ATP for uptake of [^{3}H]inositol (6.2 and 6.7% uptake of the available label, respectively). Surprisingly, under the same field strength conditions the omission of nucleotide completely from the external medium appeared to have little effect upon the % of externally available [^{3}H]inositol taken up by the platelets (results not shown). Such platelets were, however, difficult to resuspend after centrifugation and rarely showed the classical swirling birefringence seen when platelets in suspension are of single cell identity. The inclusion of 5 mM ATP was therefore adopted routinely.

Table 1

Distribution of [^{3}H]inositol in platelet phosphatidylinositides

Phospholipid	*Electropermeabilised and resealed*		
	Control non-permeabilised prep. 1 (dpm)	Prep. 2 (dpm)	Prep. 3 (dpm)
Phosphatidylinositol	928 (74.3%)	41174 (75.4%)	51142 (67.2%)
Phos. ins 4-P	194 (15.6%)	9083 (16.6%)	18214 (23.9%)
Phos. ins 4,5-P_2	126 (10.0%)	4373 (8.0%)	6802 (8.9%)

Lipids are extracted from [^{3}H]inositol-labelled platelets (3×10^8 cells) as described in section 2. Three typical preparations are shown with similar results obtained in at least 10 other preparations. Prep. 1 is non-permeabilised platelets, prep. 2 and 3 are permeabilised and resealed platelets using 7 discharges of 8.5 kV/cm. All preparations contained 8 μCi/ml [^{3}H]inositol during the uptake incubation (2 h), see section 2

Analyses of the distribution of [^{3}H]inositol incorporated into the platelet phosphoinositide pools were carried out after extraction and separation by thin layer chromatography. Table 1 shows the distribution profile for two typical experiments in which the platelets were exposed to [^{3}H]inositol during permeabilisation with 7 discharge cycles at a field strength of 8 kV/cm followed by a 2 h incubation at 37°C. Amongst the phosphoinositides, phosphatidylinositol accounted for 67–75% of the incorporated [^{3}H]inositol label with lesser amounts in phosphatidylinositol 4-phosphate (PIP, 15–24%) and in phosphatidylinositol 4,5-bisphosphate (PIP_2, 8–10%). These distribution values are broadly in keeping with the reported figures for the relative concentrations of these phosphoinositides in normal platelet membranes [8,14]. In terms of raw counts, these were between 35 and 55 times higher for the permeabilised/resealed platelet than could be achieved with control non-permeabilised platelets exposed to the same amount of [^{3}H]-inositol (table 1).

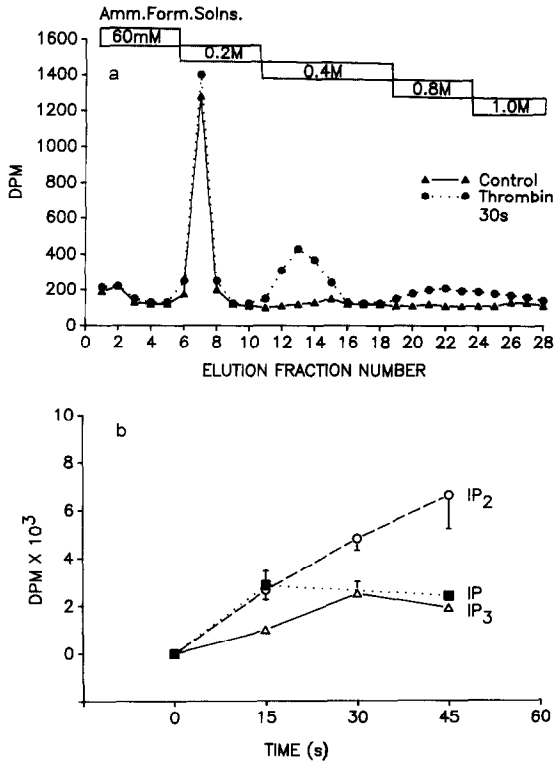

Fig.3. Thrombin-induced formation of inositol phosphates in [^{3}H]inositol-labelled permeabilised resealed platelets (7 kV/cm, 2 h incubation at 37°C). (a) Typical Dowex-formate chromatography of control (▲) and 30 s thrombin stimulated (●) production of inositol phosphates. Ammonium formate solutions in 0.1 M formic acid used to elute inositol phosphates are shown in blocks above the profiles. The control profile (no thrombin) showed no peaks of activity corresponding to IP_2 and IP_3, but an IP_1 peak was present (total counts 11 700 dpm). (b) Time course of 1 U/ml thrombin induced increase in inositol phosphate formation. Points are means ± SE (n = 3), or means of duplicate determinations, after subtraction from control incubations of platelets without thrombin treatment. Symbols represented are (△) IP_3, (○) IP_2, (■) IP_1.

Further experiments were performed in which the production of [^{3}H]inositol phosphates was monitored after exposure of [^{3}H]inositol-labelled and resealed platelets to thrombin (1 U/ml). The period of exposure to thrombin was varied between 15 and 45 s before stopping its effect with TCA (see section 2). Separation of the inositol phosphates by Dowex-formate column chromatography (fig.3a) revealed that ^{3}H-labelled IP_1, IP_2 and IP_3 were all present in detectable amounts at 15 s after addition of thrombin (fig.3b), but the relationship between the level of label associated with each of these metabolites changed substantially with increased time of exposure to thrombin. Whereas the counts for the predominant metabolite IP_2 increased approximately linearly over the 45 s, IP_3 formation peaked at 30 s and then decreased and IP formation plateaued at 15 s and was maintained at this level without significant change thereafter. In these studies lithium was not included to inhibit hydrolysis of inositol phosphate(s).

4. DISCUSSION

For studies of the metabolism of phosphoinositides and the production of inositol phosphates in human platelets a major limitation is the small amount of [^{3}H]inositol that can be incorporated into intact cells so that the formation of metabolites can be followed sequentially. Incubation periods in the presence of the label may extend to some hours and uptakes as low as 0.3–0.5% of the externally available label are commonly reported. In consequence high specific activities of externally applied [^{3}H]inositol are required in order to give reasonable counts in individually separated lipid components. There are species differences, however, for example, rabbit platelets are able to incorporate [^{3}H]inositol at higher rates than human platelets [15–17]. In this paper we report the

use of reversibly electropermeabilised platelets to introduce high concentrations of [^{3}H]inositol into normal human platelets. With as little as 8 μCi/ml [^{3}H]inositol in the externally applied medium over 6% of the available label is taken up and good incorporation occurs into the phosphoinositide pools. Our electropermeabilising unit, whose design is similar to that used by others [10,18], generates a high-voltage discharge across electrodes 3 mm apart in a chamber of about 800–1000 μl volume. Earlier investigations have shown that $\approx$7 discharge cycles at field strengths of 7–8 kV/cm gave negligible losses of lactate dehydrogenase and free diffusion into the cytosol compartment of substances of molecular mass of $\approx$600 Da [9]. Incubation at 37°C for 30–60 min encourages spontaneous resealing and the platelets' morphological and metabolic integrity are well preserved, as is their surface receptor status for activating ligands. Comparative determinations of the uptake of [^{3}H]inositol in control non-permeabilised platelets and reversibly electropermeabilised platelets from the same preparations showed that in the latter cells 10–20-fold higher amounts of the label can be taken up. Analysis of the labelled phosphoinositides PI, PIP and PIP_2 in the reversibly permeabilised platelets revealed counts between 30- and 50-fold higher for these lipids than found in extracts from control platelets. The % distribution of counts between these phospholipid species approximated those reported by other workers using much higher levels of external [^{3}H]inositol [2,4,19,20] and broadly reflected by their known proportional distribution in normal platelet membranes [14].

Studies in which the production of inositol phosphates was monitored within the first minute after exposing the prelabelled permeabilised and resealed platelets to thrombin revealed that the label incorporates into a hormone-sensitive metabolic pool. Inositol bisphosphate (IP_2) which was the predominant metabolite increased over this time course. The counts in IP_3 peaked at 30 s and decreased thereafter and the IP associated counts plateaued after 15 s. This pattern of inositol phosphate production is similar to those results reported by others where lithium is not used to inhibit IP breakdown [2]. The degradation of phosphatidylinositols is known to be induced in platelets by agonists such as thrombin and TXA_2 although there is still considerable controversy correlating Ca^{2+} mobilisation with inositol phosphate formation as a primary event for agonists such as ADP and collagen [21–23]. Thus there is much interest in investigating the origin and degradation of inositol phosphates known to be involved in intracellular Ca^{2+} mobilisation. We believe the reversible electropermeabilisation protocol outlined in this paper allows a higher incorporation of externally applied [^{3}H]inositol into platelet phospholipid pools than existing procedures. During the course of these studies a similar electropermeabilisation approach has been used by another group to achieve high incorporation of [^{3}H]inositol into the phosphoinositide pool of *Dictyostelium* cells [24].

Acknowledgements: We should like to thank Miss Heather Watson and Mrs B.J. Evenden of this Department for their assistance in the preparation of this manuscript.

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