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Phosphatidylinositol 4,5-Bisphosphate Hydrolysis in Turkey Erythrocytes Is Regulated by P_{2y} Purinoceptors

CHRISTOPHER P. BERRIE, PHILLIP T. HAWKINS, LEONARD R. STEPHENS, T. KENDALL HARDEN, and C. PETER DOWNES

Department of Cellular Pharmacology, Smith Kline and French Research Limited, Welwyn, AL6 9AR England (C.P.B., P.T.H., L.R.S., C.P.D.) and Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, NC 27514 (T.K.H.)

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SUMMARY

When intact [^{3}H]inositol-loaded turkey erythrocytes were stimulated with the purinergic agonist ADP, there was a rapid increase (2.5-fold after 30 sec) in the intracellular content of [^{3}H]inositol 1,4,5-trisphosphate, followed by increases in the levels of [^{3}H] inositol bisphosphate and [^{3}H]inositol 1,3,4,5-tetrakisphosphate (4-fold and 5-fold, respectively, after 3 min). [^{3}H]Inositol monophosphate levels did not rise in the first 3 min of ADP stimulation but increased slowly thereafter, demonstrating that the primary response of turkey erythrocytes to purinergic stimulation is hydrolysis of phosphatidylinositol 4,5-bisphosphate. Inositol phosphate accumulation was evoked by a P_{2y} purinoceptor, as indicated by the rank order of potencies of a variety of purinergic agonists. 2-Methylthioadenosine 5′-triphosphate was the most potent agonist tested, with an EC_{50} value of 0.36 μM. High performance liquid chromatography analysis demonstrated the presence of three distinct inositol tetrakisphosphate isomers in [^{3}H]inositol-loaded turkey erythrocytes, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)P_4], and inositol 3,4,5,6-tetrakisphosphate. Prolonged stimulation with adenosine 5′-*O*-(2-thiodiphosphate), a nonhydrolyzable analogue of ADP, resulted in a 60-fold increase in the level of [^{3}H]Ins(1,3,4,5)P_4, whereas a substantial rise in the [^{3}H]Ins(1,3,4,6)P_4 fraction was also seen. These results indicate that turkey erythrocytes represent a valuable model system for studies of purinoceptor function as well as fundamental aspects of cell surface receptor-regulated phosphoinositide metabolism.

Over the last few years, it has become clear that hormone stimulation of PtdIns(4,5)P_2 hydrolysis involves receptor activation of a G protein, which, in turn, activates a phosphoinositide-specific phospholipase C (PIC) (1, 2). More recently, we have proposed that turkey erythrocyte membranes may provide a valuable cell-free model for studies of guanine nucleotide activation of G protein-dependent PtdIns(4,5)P_2 hydrolysis (3). Preliminary experiments investigating potential activation of this system in [^{3}H]inositol-loaded turkey erythrocytes have shown the presence of a purinergic receptor (purinoceptor) linked to PtdIns(4,5)P_2 hydrolysis. In this paper, we describe the pharmacological characterization of the purinoceptor linked to PtdIns(4,5)P_2 hydrolysis, using as an assay the rapid accumulation of inositol phosphates in turkey erythrocytes after their exposure to purinergic agonists.

Nearly 60 years ago, Drury and Szent-Gyorgi (4) reported that adenosine elicited marked effects on cardiovascular function, and it is now known that purine nucleotides have widespread and potent extracellular actions in many tissues (see Ref. 5). In 1978, Burnstock (6) proposed a subdivision of these "purinoceptors," with adenosine and AMP preferentially stimulating P_1 receptors whereas ADP and ATP were the agonists preferred by P_2 purinoceptors. P_2 purinoceptors were further divided into P_{2x} and P_{2y} subtypes based upon comparative studies of ADP and ATP analogue potencies (see Ref. 7). This classification has been further supported by more recent studies (see Ref. 5) and by experiments aimed at describing the signal transduction mechanisms employed by purinoceptor subtypes. Thus, the responses of many cell types to ATP appear to involve both the mobilization of intracellular calcium and PtdIns(4,5)P_2 hydrolysis. These include isolated hepatocytes (8–13), endothelial cells (14, 15), Ehrlich ascites tumor cells (16, 17), and rat aortic myocytes (18). In the present study, we demonstrate that turkey erythrocytes possess a population of

ABBREVIATIONS: PtdIns(4,5)P_2, D-phosphatidyl-myo-inositol-4,5-bisphosphate; PtdIns, D-phosphatidyl-myo-inositol; PtdIns(4)P, D-phosphatidyl-myo-inositol 4-phosphate; $InsP_2$, $InsP_3$, $InsP_4$, inositol polyphosphates of unknown isomerism; Ins(1,4)P_2, D-myo-inositol 1,4,-bisphosphate, similar abbreviations are used for all inositol phosphate isomers of known conformation; AMPS, adenosine 5′-*O*-thiomonophosphate; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPYS, adenosine 5′-*O*-(3-thiotriphosphate); SpATPαS, adenosine 3′,5′-monophosphothionate, cyclic Sp isomer; 2MeSATP, 2-methylthioadenosine 5′-triphosphate; HomoATP, 5′-deoxy-5′((hydroxypyrophosphoroxy)phosphinylmethyl)-adenosine; AMPPNP, adenylylimidodiphosphate; AMPPCP, adenylyl(β,γ-methylene)-diphosphonate; AMPCPP, adenylyl(α,β-methylene)-diphosphonate; PIC, phosphoinositidase C, phosphoinositide-specific phospholipase C; HEPES, 4-(2-hydroxylethyl)-1-piperazineethanesulphonic acid; HPLC, high performance liquid chromatography; G protein, guanine nucleotide-dependent protein.

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P_{2y} purinoceptors whose activation, by a variety of purinergic agonists, stimulates the hydrolysis of PtdIns(4,5)P_2. In addition the products of this reaction have been studied both by conventional ion exchange chromatography (19) and by recently developed HPLC techniques (20) that allow resolution of a variety of inositol phosphate isomers. This has enabled us to study the metabolic relationship between the fast signalling events initiated by PtdIns(4,5)P_2 hydrolysis and synthesis of inositol phosphates, such as inositol pentakisphosphate, the metabolic origins of which are currently unknown.

Materials and Methods

[^{3}H]inositol loading of turkey erythrocytes. Turkey blood was either collected from one to three freshly killed turkeys (250 ml) and mixed with 1 ml of heparin sodium (5000 units; Evans Medical Ltd.) or withdrawn from the brachial vein (30–60 ml) of a single live turkey and mixed with 100 μl of heparin sodium (500 units). Washed erythrocytes were prepared from whole blood by the method described previously (21), with the final washes being carried out in Eagles Medium (GIBCO, Uxbridge, Middx) supplemented with 25 mM Na HEPES and with the pH adjusted to 7.3 (Eagles/HEPES).

Before being added to the washed cells, 0.5 μl (0.5 mCi) of [^{3}H] inositol (NEN, Stevenage, Kerts) in ethanol/water (9:1) was evaporated to dryness under a stream of nitrogen gas. The [^{3}H]inositol was then dissolved in 200 μl of deionized water and passed through a 200-μl Dowex AG1x8 (200–400 mesh; formate form; Bio-Rad, Watford, Kerts) column in order to remove polar contaminants. [^{3}H]Inositol loading of washed turkey erythrocytes was carried out in a medium containing 4 ml of washed, packed cells, 16 ml of Eagles/HEPES, and 0.5 mCi of [^{3}H]inositol (prepared as described above). The cells were incubated for 3 hr (unless otherwise stated) at 37° in a shaking water bath, with the atmosphere above the incubation being replenished with 95% oxygen 5% carbon dioxide every hour. The [^{3}H]inositol loading was terminated by centrifugation at 1000 × *g* for 5 min with the packed cells being washed an additional two times in warm (37°) Eagles/ HEPES.

Stimulation of [^{3}H]inositol-loaded cells. After the final wash, the packed [^{3}H]inositol-loaded cells were resuspended to 20–30 ml in warm Eagles/HEPES and incubated for 10 min at 37° in a shaking water bath before drug additions were made. To determine time courses of inositol polyphosphate accumulation in response to purinergic agonists, a 50× concentrated solution of the agonist in Eagles/HEPES (pH adjusted to 7.3) was added to the loaded cells at 37°; 200-μl aliquots were then removed at the given times and added to 250 μl of cold (4°) 10% perchloric acid. In order to obtain agonist dose-response curves, 200-μl aliquots of the loaded cells were added to 50 μl of 5× concentrated ligand solutions in Eagles/HEPES (pH adjusted to 7.3) at 37°. These incubations were terminated after 60 sec by addition of 250 μl of cold 10% perchloric acid. The [^{3}H]inositol phospholipids and [^{3}H]inositol phosphates present in these incubations were extracted and separated by methods described previously (19, 22), with final quantitation by liquid scintillation counting. Pharmacological data were fitted to a four-parameter logistic equation using nonlinear least squares regression analysis (23).

HPLC analysis of inositol phosphates. Recently delineated HPLC techniques (20) were used to separate the various inositol polyphosphate isomers that were present in turkey erythrocytes at given times after stimulation. This involved the addition of EGTA (100 mM; 25 μl; Fluka) and ^{32}P-labeled standards (1000–3000 cpm; prepared exactly as described in Ref. 20) to the upper phase of tri-*n*-octylamine/ Freon (Aldrich/BDH)-extracted samples (700 μl; see Ref. 19). After filtration (0.2 μm), the samples were injected onto a weak anion-exchange HPLC column (Partisphere WAX, 5-μm particle size in a 12.5 cm cartridge; Whatman, Maidstone, Kent) and the various inositol phosphates were eluted with a gradient delivered from two independent pumps drawing on reservoirs containing H_2O and 0.25 M $(NH_4)_2HPO_4$ (pH 3.2 with H_3PO_4 at 25°; BDH), respectively, at a flow rate of 1 ml/ min (exactly as described in Ref. 20). Liquid scintillation cocktail (Instagel; Packard, Groningen, The Netherlands) was added to the resulting fractions (0.6 min/fraction) and the levels of ^{3}H- and ^{32}P-containing compounds were determined by dual-label liquid scintillation counting.

Purinergic ligands. The majority of the purinergic ligands used in the study were obtained from Boehringer Corporation Ltd. However, our thanks go to Dr. W. Howson (Smith Kline & French Research Ltd, Welwyn) for purity tests and initial supplies of 2MeSATP (later purchased from Research Biochemicals), AMPCPP (later purchased from P-L Biochemicals), HomoATP, AMPPCP, AMPS, and adenosine (the last four compounds originating from Sigma Chemical Co., Poole, Dorset).

Results

[^{3}H]inositol-loaded turkey erythocytes. We have previously demonstrated (3, 21) the presence of a G protein-regulated PIC in turkey erythrocyte membranes prepared from cells that had been incubated with [^{3}H]inositol for a period of 18–24 hr. Preliminary experiments (21) indicated that these membranes may contain a receptor responsive to ATP. We have now assessed the ability of a variety of purinergic agonists to stimulate PtdIns(4,5)P_2 hydrolysis when applied to intact [^{3}H] inositol-loaded turkey erythrocytes.

Fig. 1 demonstrates the rate of incorporation of [^{3}H]inositol into PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 of intact turkey erythrocytes under the modified conditions used in the present study (see Materials and Methods). Although the phosphoinositide pools appeared to be approaching equilibrium labeling after 20–25 hr, at this time there were also substantial levels of ^{3}H-labeled polar inositol phosphates, corresponding to the inositol tetrakis- and pentakisphosphate fractions (after 4–6 hr of incubation; see Fig. 2). Preliminary experiments (data not shown) indicated that these high levels of ^{3}H radioactivity in the inositol polyphosphate pools of unstimulated cells that had been labeled for 20 hr greatly obscured the observation of

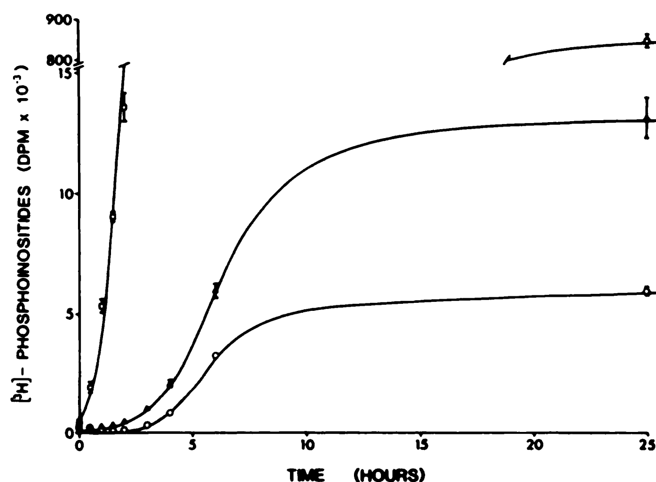

Fig. 1. Incorporation of [^{3}H]inositol into the inositol phospholipids of the turkey erythrocyte. Cells were washed and incubated with 0.5 mCi of [^{3}H]inositol in an Eagles/HEPES medium at 37° (see Materials and Methods). At the indicated times, samples of the incubation were added to 10% perchloric acid and the [^{3}H]inositol phospholipids were extracted and separated as described in Ref. 22, enabling the levels of [^{3}H] phospholipids to be determined. □, PtdIns; Δ, PtdIns(4)P; and O, PtdIns(4,5)P_2. Values shown are means ± standard errors for triplicate determinations.

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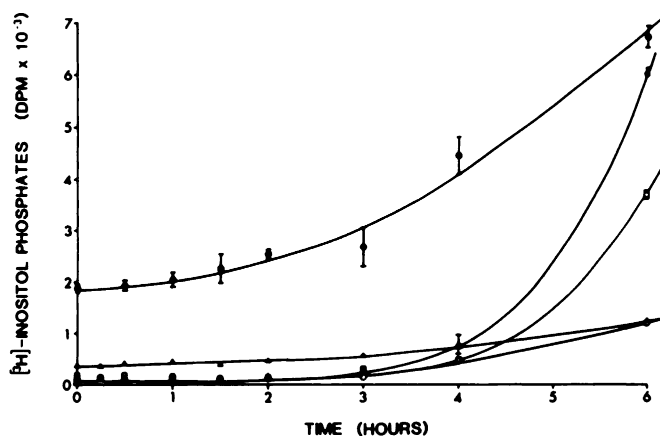

Fig. 2. [^{3}H]Inositol labeling of inositol phosphates in unstimulated turkey erythrocytes. Cells were washed and incubated with 0.5 mCi of [^{3}H] inositol in an Eagles/HEPES medium at 37° (see Materials and Methods). At the indicated times, samples of the incubation were added to 10% perchloric acid and the [^{3}H]inositol phosphate levels were determined. ●, InsP; ▲, $InsP_2$; ○, $InsP_3$; △, $InsP_4$; and □, $InsP_5$. Values shown are means ± standard errors for triplicate determinations.

agonist-induced accumulation of inositol phosphates. When the labeling period was reduced to 3 hr, allowing substantial labeling of the phosphoinositides, very low background levels of ^{3}H radioactivity were maintained in the inositol polyphosphates (see Figs. 1 and 2). It was also possible to reduce the apparent level of [^{3}H]InsP seen at time zero (see Fig. 2) by washing of the cells before agonist stimulation (see Materials and Methods), thus reducing any ^{3}H-polar contaminants and any carry-over of the high levels of [^{3}H]inositol used in the loading incubations. These labeling conditions allowed agonist-induced accumulation of the inositol phosphates to be readily observed (see below).

Characteristics of ADP-stimulated inositol phospholipid hydrolysis. The time courses of accumulation of inositol phosphates after ADP stimulation of intact turkey erythrocytes that had been incubated with [^{3}H]inositol for 3 hr at 37° are illustrated in Fig. 3. The primary response of these cells to ADP application was an initial rapid increase in the [^{3}H]$InsP_3$ fraction eluted from Dowex columns (see Materials and Methods), peaking within the first 30–40 sec of stimulation. This was followed by a second slower increase in [^{3}H]$InsP_3$ levels. The nature of this biphasic response was further investigated by HPLC analysis of [^{3}H]inositol phosphate content after ADP stimulation (see below). The levels of ^{3}H radioactivity associated with the $InsP_2$ and $InsP_4$ fractions, both of which reached their peak 2–3 min after ADP stimulation, showed a slower initial increase than that seen with [^{3}H]$InsP_3$ (see Fig. 3). [^{3}H] $InsP_5$ levels remained unchanged within the first 20 min after ADP stimulation (data not shown) whereas [^{3}H]InsP levels accumulated only very slowly (see Fig. 3).

Pharmacology of purinoceptor-mediated inositol phospholipid hydrolysis. The pharmacological character of the purinoceptor mediating inositol phospholipid hydrolysis has been determined by measuring the [^{3}H]inositol phosphates ($InsP_2$ plus $InsP_3$ plus $InsP_4$) that accumulated after stimulation of the cells for 60 sec at 37° with a variety of purinergic agonists. This strategy yielded a readily measured signal and minimized the impact of agonist degradation (see below) upon the position and shape of the observed dose-response curves. Furthermore, HPLC analysis (see below) revealed that the radioactivity that accumulated in these fractions during the first minute of stimulation was restricted to Ins(1,4,5)P_3[1] and its previously decribed metabolites, Ins(1,3,4,5)P_4, Ins(1,3,4)P_3, and Ins(1,4)P_2.

Fig. 4 illustrates the dose-response curves obtained under these conditions for a natural agonist (ADP), a metabolically stable analogue (ADPβS), and the most potent agonist identified in these studies (2MeSATP). Table 1 contains a summary of the collected data from stimulation of turkey erythrocytes by a variety of potential purinergic agonists. Neither AMP nor adenosine were effective agonists and the rank order of potency of agonists (shown in Table 1) was 2MeSATP > ADPβS > AMPPNP > ATP = AMPCPP = ADP $\gg$ AMPPCP. Most notably, the P_{2y}-selective agonist 2MeSATP (which is approximately equipotent with ATP when acting on the P_{2x} receptor) is some 250-fold more potent than ADP itself. Table 1 also shows that a number of the purinergic agonists were unable to elicit inositol phosphate accumulation to the same maximal extent as ADP. Secondly, a common feature of the effects of ATP and its analogues on turkey erythrocytes was a suppression of inositol phosphate accumulation at ligand concentrations ranging from 10- to 100-fold above their EC_{50} values (data not shown). Although these two effects may well be linked, no further explanation of the underlying mechanisms can be arrived at from the data available at present.

HPLC analysis of inositol phosphates in turkey erythrocytes. Chromatography of inositol phosphates on small Dowex-1 anion-exchange columns is incapable of separating inositol phosphate isomers that differ only in the distribution of phosphate groups around the inositol ring (24). In order to gain further information on the [^{3}H]inositol-labeled compounds present in fractions eluted from Dowex columns, a number of samples from agonist-stimulated turkey erythrocytes were produced in parallel with those subjected to Dowex column chromatography and were analyzed instead by weak anion-exchange HPLC after being spiked with ^{32}P-labeled inositol polyphosphate standards (see Materials and Methods). An example of such an analysis is illustrated in Fig. 5, showing the presence of different [^{3}H]$InsP_3$ and [^{3}H]$InsP_4$ isomers in a sample after a 30-sec stimulation with ADP. A comparison of this data and that obtained after a 10-min stimulation with ADP (see Table 2) shows that, at early times of ADP stimulation, the predominant [^{3}H]$InsP_3$ isomer is [^{3}H]Ins(1,4,5)P_3. At the longer time point, this initial increase in [^{3}H]Ins(1,4,5)P_3 has reversed, with the major [^{3}H]$InsP_3$ isomer now being [^{3}H] Ins(1,3,4)P_3. These data support the concept that the biphasic nature of the time course of [^{3}H]$InsP_3$ production (see Fig. 3) is mainly due to an initial transient increase in the level of [^{3}H] Ins(1,4,5)P_3, whereas at longer times the dominant product is the [^{3}H]Ins(1,3,4)P_3 isomer. Furthermore, although three different $InsP_4$ isomers have recently been described (24–27), the ADP-stimulated increase in the [^{3}H]$InsP_4$ fraction was confined almost entirely to a single isomer, Ins(1,3,4,5,)P_4.

As shown in Fig. 3 and Table 2, the levels of radioactive $InsP_2$, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 reached their peaks within the first few minutes after exposure to ADP, followed by a slow decline. This suggested either a rapid desensitisation

[1] The D-forms of the inositol phosphate isomers and inositol phospholipids are used throughout. In all cases bar one (D-Ins(3,4,5,6)P_4 used = L-Ins(1,4,5,6)P_4 recommended), this agrees with the nomenclature system recommended by the IUPAC-IUB commission on Biochemical nomenclature (1976).

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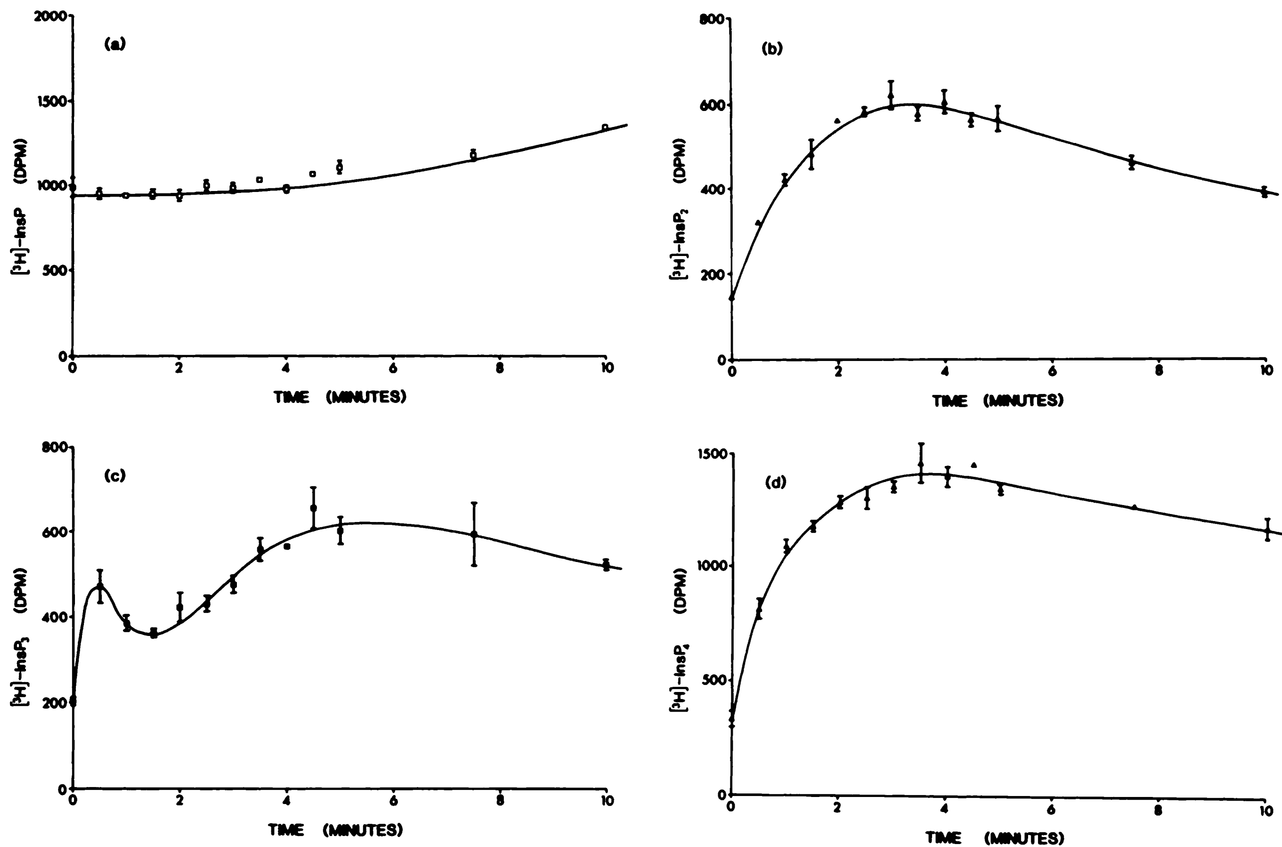

Fig. 3. Time courses for ADP-stimulated production of [^{3}H]inositol phosphates in [^{3}H]inositol-loaded turkey erythrocytes. Cells were washed and incubated with [^{3}H]inositol for 3 hr in an Eagles/HEPES medium at 37° (see Materials and Methods). After an additional two washes in warm [^{3}H] inositol-free medium and a 10-min preincubation at 37°, ADP was added to a final concentration of 1 mM and samples of the incubation were added to 10% percholic acid at the indicated times. The [^{3}H]inositol phosphates were extracted and separated as described in Ref. 19, enabling time courses for ADP stimulation of the production of InsP (a), InsP$_2$ (b), InsP$_3$ (c), and InsP$_4$ (d) to be determined. Values shown are means ± standard errors for triplicate determinations.

of the purinoceptor-mediated phosphoinositide hydrolysis or degradation of the ADP to inactive AMP and adenosine (see Table 1) by ectonucleotidases (28). The latter possibility was tested by readdition of a second pulse of ADP (to 1 mM) 5 min after the initial stimulation, as illustrated in Fig. 6. This second pulse of ADP caused a further accumulation of inositol phosphates almost identical in both magnitude and time course to the initial response, suggesting that the original ADP had indeed been hydrolyzed. This conclusion was further supported by comparing the response to ADP with that of a nonhydrolyzable analogue, ADPβS. Although this compound was just as effective as ADP when tested using a 60-sec exposure to each agonist (see Table 1 and Fig. 4), the accumulation of total [^{3}H] inositol polyphosphates (InsP$_2$–InsP$_5$ combined) after prolonged exposure to ADPβS was quite different from that of ADP. That is, the accumulation of [^{3}H]inositol polyphosphates during treatment with ADPβS eventually greatly exceeded that evoked by ADP. Thus, after 30 min of stimulation with ADPβS, there was a 7-fold increase in the radioactivity accumulating in the combined inositol polyphosphate fraction, in comparison with samples that had been stimulated for the same time with ADP (Fig. 7). HPLC analysis of these samples stimulated for 30 min with ADPβS (Table 2) demonstrated that most of the increase observed in the InsP$_4$ fraction coincided with a [^{32}P] Ins(1,3,4,5,)P$_4$ standard. At this time, however, approximately 15% of the radioactivity in the InsP$_4$ region of the chromatogram comigrated with a [^{32}P]Ins(1,3,4,6,)P$_4$ standard, amounting to a 15-fold increase over the levels seen in the absence of ADPβS. Although at this time there was little or no change in [^{3}H]InsP$_5$ levels, they did increase by some 3-fold above control after 60 min of stimulation in the presence of ADPβS, a situation that was not seen with the transient stimulation caused by ADP (data not shown). The significance of these increases in the higher inositol polyphosphates will be discussed later with reference to recent work on other cell types.

Discussion

Previous studies have shown that turkey erythrocyte membranes contain a PIC activity that is regulated by guanine nucleotides (3, 21), implicating a specific G protein in the process of hormone-stimulated PtdIns(4,5)P$_2$ hydrolysis. Initial experiments with intact turkey erythrocytes suggested that they also possess a population of purinergic receptors that are coupled to PtdIns(4,5)P$_2$ hydrolysis. We have now characterized this response with respect to the pharmacological specificity of the receptor and the complex spectrum of inositol phosphates that accumulate in the intact cells after purinergic stimulation.

The absence of specific purinoceptor antagonists has led to a classification of these receptors based upon the rank orders of potency of a variety of purinergic agonists (7). When applied to PtdIns(4,5)P$_2$ hydrolysis in turkey erythrocytes, this type of analysis indicates the participation of a P$_{2y}$ purinoceptor (see

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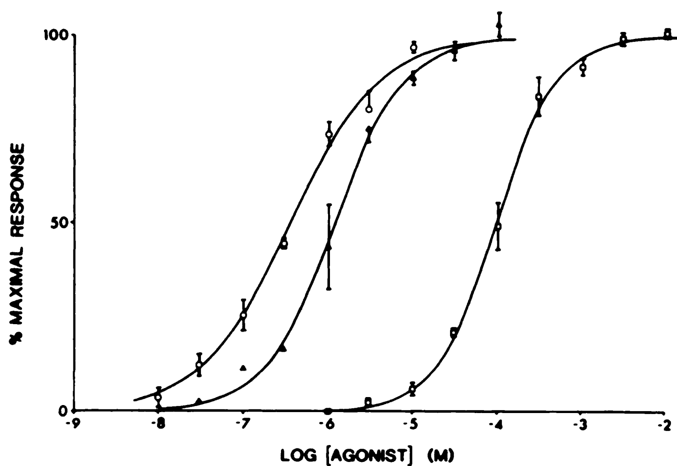

Fig. 4. Agonist dose-response curves for stimulation of [^{3}H]inositol polyphosphate production in turkey erythrocytes. Cell preparation and sample extraction were exactly as described in the legend to Fig. 3, with the final separation involving the pooling of the $InsP_2$, $InsP_3$, and $InsP_4$ fractions from the Dowex columns after a 60-sec incubation of the [^{3}H] inositol-loaded cells with 2MeSATP (○), ADPβS (△), and ADP (□). Data were fitted to a four-parameter logistic equation using nonlinear least squares regression analysis and the values shown represent the mean ± standard errors of three separate determinations using different blood samples (each agonist concentration being assayed in triplicate). The stimulation of [^{3}H]inositol polyphosphate production is expressed as a percentage of the predicted maximum from the fitted data in each experiment. The *curves* are those computed as the best fits of the values shown.

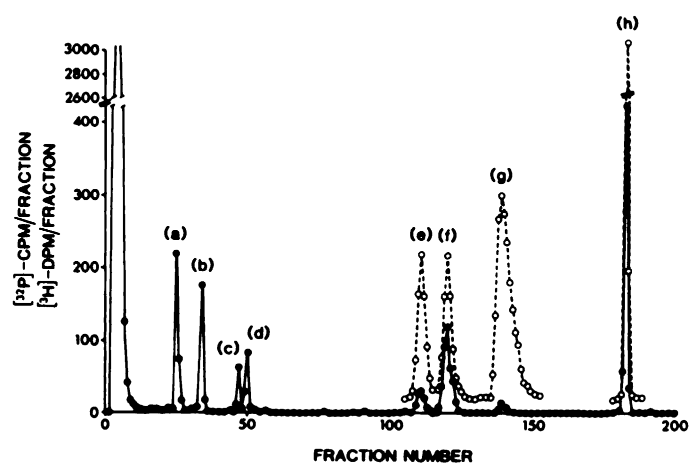

Fig. 5. HPLC separation and identification of the [^{3}H]inositol phosphates produced after 30 sec of ADP stimulation of [^{3}H]inositol-loaded turkey erythrocytes. Cell preparation and sample extraction were exactly as described in the legend to Fig. 3, with the final separation being carried out on a Partisphere WAX column (see Materials and Methods). The levels of ^{3}H radioactivity (●) associated with the higher inositol phosphates ($InsP_4$ and $InsP_5$) co-eluted with ^{32}P-labeled standards (○) of known isomeric configuration (Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(3,4,5,6)P_4, and Ins(1,3,4,5,6)P_5, prepared exactly as described in Ref. 20). Identification of the other ^{3}H-labeled compounds (InsP, $InsP_2$, and $InsP_3$) was determined from the co-elution with known ^{32}P-labeled standards under the same conditions of sample preparation, application, and elution. These [^{3}H]inositol phosphates are: *a*, InsP; *b*, $InsP_2$; *c*, Ins(1,3,4)P_3; *d*, Ins(1,4,5)P_3; *e*, Ins(1,3,4,6)P_4; *f*, Ins(1,3,4,5)P_4; *g*, Ins(3,4,5,6)P_4; and *h*, Ins(1,3,4,5,6)P_5. Values are determinations of the radioactivity associated with each fraction with background levels between peaks being shown as the means of two sequential fractions.

TABLE 1

Pharmacological profile of turkey erythrocyte purinoceptors linked to PIC

Turkey erythrocytes were washed and incubated with [^{3}H]inositol for 3 hr in an Eagles/HEPES medium at 37° (see Materials and Methods). After an additional two washes and a 10-min preincubation at 37°, the various potential purinergic agonists were added to a range of concentrations that did not exceed 10^{-2} M. These incubations were terminated after a further 60 sec at 37° and [^{3}H]inositol phosphates were analyzed as described in Materials and Methods. The data represent the radioactivity in the pooled $InsP_2$ plus $InsP_3$ plus $InsP_4$ fractions and were fitted to a four-parameter logistic equation using nonlinear least squares regression analysis; the values shown represent the means of three separate determinations using different blood samples (each ligand concentration being assayed in triplicate).

Ligand	EC_{50}	Maximum response[a]
	μM	%
ADP	94.3	100
AMPCPP	71.1	38
ATP	58.2	80
SpATαS	32.4	105
AMPPNP	25.5	104
HomoATP	21.6	65
ATPYS	11.9	100
ADPβS	1.20	133
2MeSATP	0.36	122
AMPPCP	NR[b]	0
AMPS	NR	0
Adenosine	NR	0

[a] Expressed as a percentage of the maximal response to ADP.
[b] NR, no response obtained up to 10^{-2} M.

TABLE 2

Inositol phosphates produced during purinergic stimulation of turkey erythrocytes

HPLC separation and identification of the [^{3}H]inositol phosphates produced by agonist stimulation of washed, [^{3}H]inositol-loaded turkey erythrocytes (prepared as described in Materials and Methods) at 37°. ADP stimulation of 30 sec and 10 min demonstrates the changes in the relative levels of the $InsP_3$ isomers over this time period, whereas the effects of the nonhydrolyzable agonist ADPβS after 30 min of stimulation are seen in the profile of $InsP_3$ and $InsP_4$ isomers produced (see text). Over these times of stimulation, the control levels of the various [^{3}H]inositol phosphates were seen to remain essentially unchanged. Under each of the four conditions, five replicate assays were run in parallel, two of which were pooled for injection onto a Partisphere WAX column; sample preparation, application, and elution was as described in Materials and Methods. The remaining three samples were analyzed independently by batch elution from Dowex columns as described in Materials and Methods. These samples, in which inositol polyphosphate isomers were not separated, gave data that were in close agreement with those shown in the table.

Inositol phosphate	Control	ADP 30 sec	ADP 10 min	ADPβS, 30 min
		dpm		
InsP	415	331	749	1643
$InsP_2$	63	242	192	575
Ins(1,3,4)P_3	84	92	258	1196
Ins(1,4,5)P_3	16	142	47	466
Ins(1,3,4,6)P_4	49	125	113	759
Ins(1,3,4,5)P_4	93	406	501	5134
Ins(3,4,5,6)P_4[a]	66	33	30	11
Ins(1,3,4,5,6)P_5	402	536	304	576

[a] D-form of $InsP_4$ used throughout = L-Ins(1,4,5,6)P_4.

Table 1) with the most significant finding being that the P_{2y}-selective agonist 2MeSATP is some 250-fold more potent than either ATP or ADP. The overall rank order of potencies for a variety of purinergic agonists also agrees well with that expected for a P_{2y} receptor as defined by Burnstock and Kennedy (7).

Receptor subtypes may also be classified on the basis of the signal transduction pathways that they employ. P_1 receptors have been known for some time to be involved in the regulation of adenylate cyclase (stimulation via A_1 and inhibition via A_2 receptor subtypes, respectively) (29). However, it was not until the early 1980s that P_2 receptors were shown to be linked to

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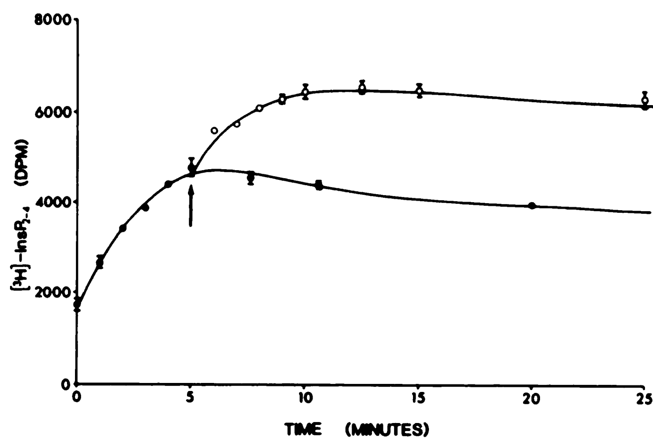

Fig. 6. ADP stimulation of [^{3}H]inositol polyphosphate production in turkey erythrocytes; effect of ADP readdition. Cell preparation and sample extraction were exactly as described in the legend to Fig. 3, with the final separation involving the pooling of the $InsP_2$, $InsP_3$, and $InsP_4$ fractions from Dowex columns (see text). The control curve for ADP (1 mM) stimulation (●) is shown, with a second pulse of ADP (1 mM) being added (see *arrow*; ○) 5 min after the initial ADP addition. Values shown are means ± standard errors for triplicate determinations.

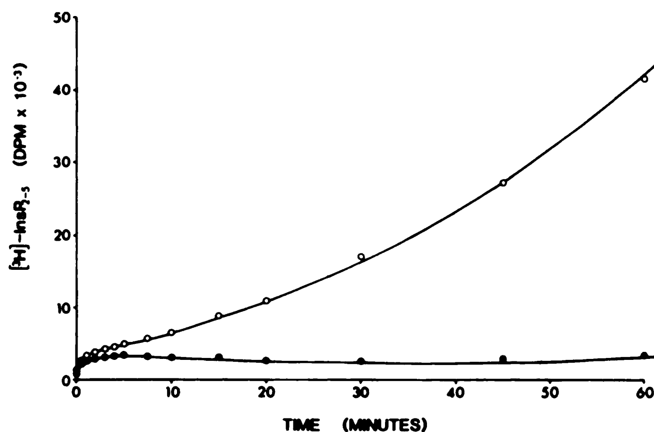

Fig. 7. ADP (●) and ADPβS (○) stimulation of [^{3}H]inositol polyphosphate production in turkey erythrocytes. Cell preparation and sample extraction were exactly as described in the legend to Fig. 3, with the final separation involving the pooling of the $InsP_2$, $InsP_3$, $InsP_4$, and $InsP_5$ fractions from Dowex columns (see text). Values shown are means ± standard errors for triplicate determinations.

calcium mobilization and PtdIns(4,5)P_2 hydrolysis, with the production of $InsP_3$ being detected in various cell types (11–13, 15, 18). Although the available pharmacological data in all of these studies is relatively limited, it does indicate the probable participation of the P_{2y} receptor subtype in these responses. The situation has been complicated further by a recent study (13) that suggested that the hepatocyte P_2 receptor may independently interact with two signal transduction systems, namely, the stimulation of PtdIns(4,5)P_2 hydrolysis via a pertussis toxin-insensitive mechanism and the inhibition of adenylate cyclase via the pertussis toxin-sensitive G protein (N_i). Moreover, the rank orders of potency of some of the agonists used in this study gave limited support for the notion that both these responses involved P_{2y} receptors. Those data may indicate that a single receptor subtype functions through distinct transduction pathways or, perhaps more likely, that the limited spectrum of discriminating purinergic agonists used in this study failed adequately to distinguish between distinct P_2 receptor subtypes.

Several lines of evidence indicate that receptor-dependent hydrolysis of phosphoinositides primarily involves the specific cleavage of PtdIns(4,5)P_2 to give Ins(1,4,5)P_3 and 1,2-diacylglycerol (30). Both of these products act as second messengers, in the mobilization of intracellular calcium and the activation of protein kinase C, respectively. As expected for an intracellular signal molecule, Ins(1,4,5)P_3 is rapidly metabolized by two distinct pathways, by 5-specific dephosphorylation to Ins(1,4,)P_2 (31) and by phosphorylation to Ins(1,3,4,5,)P_4 (32, 33). The latter compound has been implicated in hormone regulation of the entry of extracellular calcium into cells under certain circumstances (34, 35) and is also degraded by 5-phosphomonoesterase activity to give a second $InsP_3$ isomer, Ins(1,3,4,)P_3 (22, 36). The pattern of inositol phosphate formation observed in ADP-stimulated turkey erythrocytes is entirely consistent with the existence of these pathways in such cells. Thus, HPLC analysis revealed that Ins(1,4,5)P_3 accounted for most of the $InsP_3$ that accumulated very rapidly after stimulation, whereas Ins(1,3,4)P_3 was the dominant isomer after prolonged exposure to agonists (see Table 2). This initial accumulation of Ins(1,4,5)P_3 was also followed soon afterwards by the formation of Ins(1,3,4,5)P_4 and an $InsP_2$ isomer that we have not specifically identified (although evidence suggests that the major $InsP_2$ isomer formed initially is Ins(1,4)P_2[2]. Furthermore, both $InsP_3$ kinase and $InsP_3$ 5-phosphatase activities have been observed in turkey erythrocyte membranes (37).

Analysis of changes in the inositol phospholipids on ADP stimulation also demonstrated that the PIC in these cells is primarily linked to the hydrolysis of PtdIns(4,5)P_2, apparently with no direct breakdown of PtdIns itself (data not shown).

The pathways of Ins(1,4,5)P_3 metabolism have been further complicated by the recent detection of two novel $InsP_4$ isomers. Ins(1,3,4,6,)P_4 was initially described as a product of Ins(1,3,4,)P_3 phosphorylation in hepatocyte homogenates (25) and subsequently as a component of several mammalian and avian cell types (20), whereas Ins(3,4,5,6)P_4 was identified as the most prominent $InsP_4$ isomer in unstimulated macrophages and avian erythrocytes (26). Furthermore, both of these novel $InsP_4$ isomers may be further phosphorylated to Ins(1,3,4,5,6)P_5, a component present at very high concentrations in avian erythrocytes (20, 27).

In the present study (see Table 2), we have demonstrated that Ins(1,3,4,5)P_4 is the major $InsP_4$ isomer that accumulates in agonist-stimulated turkey erythrocytes. Prolonged incubation with ADP did not further enhance the accumulation of any of the inositol phosphates, due to rapid hydrolysis of the ligand. However, very substantial increases in each of the inositol phosphate pools were achieved with prolonged incubation with ADPβS (see Table 2 and Fig. 7). Even after 30 min of stimulation with the nonhydrolyzable analogue ADPβS, the major $InsP_4$ isomer was still Ins(1,3,4,5)P_4. However, at this time there was also a substantial increase (approximately 15-fold) in the level of Ins(1,3,4,6,)P_4 (see Table 2). At longer incubation times, there was also enhanced labeling of the $InsP_5$ fraction (40–60 min with 100 μM ADPβS; data not shown). These results indicate that a major pathway for the removal of

[2] L. Stephens, unpublished results.

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$Ins(1,4,5)P_3$ in these cells involves its phosphorylation to $Ins(1,3,4,5)P_4$, followed by dephosphorylation to $Ins(1,3,4)P_3$. The fate of this compound appears to be 2-fold, because it is rapidly degraded in many cells (24) and may also be involved with the synthesis of $InsP_5$. $Ins(1,3,4)P_3$-6-kinase and $Ins(1,3,4,6)P_4$ 5-kinase activities have been identified in a rat brain homogenate and soluble fraction (20), and more recent investigations have also shown them to exist in avian erythrocytes.[2] Thus, their presence in these systems would be consistent with a link between prolonged ADPβS stimulation of $PtdIns(4,5)P_2$ hydrolysis and the large increases in the $Ins(1,3,4,6)P_4$ and $InsP_5$ levels seen.

In summary, turkey erythrocytes possess a population of P_{2y} purinoceptors linked via a G protein to $PtdIns(4,5)P_2$ hydrolysis. They also have a complex pattern of inositol phosphates, many of which appear to be either metabolites of $Ins(1,4,5)P_3$ or intermediates in the biosynthesis of $InsP_5$. The latter compound is present at very high concentrations in avian erythrocytes (it may regulate the binding of oxygen to haemoglobin, fulfilling the same function as 2,3-bisphosphoglycerate in mammalian erythrocytes) and has recently also been found as a component of several mammalian tissues (20). Although, at this stage, it is not possible to deduce the full significance of these interconnecting inositol polyphosphate pathways within the cell, turkey erythrocytes may serve as an ideal model system in which to study both the molecular details of purinoceptor function and the complex cellular metabolism of inositol phosphates.

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Send reprint requests to: Christopher P. Berrie, Department of Cellular Pharmacology, Smith Kline and French Research Limited, The Frythe, Welwyn, Hertfordshire, AL6 9AR England.

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