RESEARCH COMMUNICATION

Photoaffinity labelling of cyclic GMP-binding proteins in human platelets

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The photoaffinity labelling of platelet cyclic GMP (cGMP)-binding proteins by [³²P]cGMP was studied; at least five labelled proteins (110, 80, 55, 49 and 38 kDa) were detected in platelet cytosol and four (80, 65, 49 and 38 kDa) in platelet membranes. The 110 kDa species was identified as cGMP-inhibited cyclic AMP (cAMP) phosphodiesterase (PDE III) by immunoprecipitation and by the inhibition of photolabelling by specific inhibitors of this enzyme. Similarly, the 80 kDa species was identified as cGMP-dependent protein kinase by immuno-

precipitation and by the effects of cGMP analogues on photolabelling. Addition of cAMP greatly enhanced the labelling of this 80 kDa protein, implying the existence of a potentially important interaction between the effects of cGMP and cAMP. The 65 kDa photolabelled protein appears to be a novel platelet cyclic-nucleotide-binding protein. In contrast, the 49 and 55 kDa photolabelled species are probably the RI and RII regulatory subunits of cAMP-dependent protein kinase, and the 38 kDa protein(s) may be proteolytic fragment(s) of RI and/or RII.

INTRODUCTION

Cyclic GMP (cGMP) is believed to mediate the inhibition of blood platelet function by nitric oxide and drugs that release nitric oxide [1]. Although direct activation of cGMP-dependent protein kinase cGMP-PK is likely to play a major role in this action of cGMP [2,3], there is increasing evidence that cGMP can also exert inhibitory effects in platelets by preventing the breakdown of cyclic AMP (cAMP) by the cGMP-inhibited cAMP phosphodiesterase (PDE III) [4]. In addition, cGMP exerts major effects in some cells through activation of cGMP-stimulated cyclic nucleotide phosphodiesterases (PDE II) [5] or by direct modulation of ion channels [6,7]. These considerations, and the possible existence of as-yet-unidentified targets for cGMP, indicate a need for a general method of labelling cGMP-binding proteins in cell extracts.

Direct photoaffinity labelling of unidentified cellular proteins by [³H]cGMP has been documented and apparently resulted from photoactivation of aromatic amino acids, rather than of a cGMP [8,9]. In experiments with [³²P]cGMP, Groppi et al. [10] observed photoaffinity labelling of the RI subunit of cAMP-dependent protein kinase and of PDE III in K30a lymphoma cells, which overexpress the latter enzyme. In addition, labelling of two platelet proteins of 93 kDa and 78 kDa has been reported in abstract form [11]. Apart from these studies, photoaffinity labelling by [³²P]cGMP appears to have been used exclusively to label purified cGMP-binding proteins, specifically PDE II [12,13] and the cGMP-binding cGMP phosphodiesterase (PDE V) [14]. The first objective of the present work was thus to optimize the photoaffinity labelling of platelet cytosolic and membrane proteins by [³²P]cGMP, so as to detect as many known and novel cGMP-binding proteins as possible. We then investigated factors that affected the photoaffinity labelling of individual proteins and identified some of these labelled species.

EXPERIMENTAL

Materials

[³²P]cGMP (1500 Ci/mmol) was obtained from ICN Biomedicals Canada Ltd. (Mississauga, ON, Canada). Heparin, leupeptin, phenylmethanesulphonyl fluoride, Protein A-Sepharose, cGMP, cAMP, 8-bromo-cGMP, 3-isobutyl-1-methylxanthine (IBMX), Nonidet P40 and protein standards (myosin heavy chain, 205 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa) were obtained from Sigma (St. Louis, MO, U.S.A.). Rp-8-bromo-cGMPs, Sp-cAMPS and Rp-cAMPS (see the abbreviations footnote for definition) were supplied by the BioLog Life Science Institute (La Jolla, CA, U.S.A.). Samples of bovine lung cGMP-PK (type 1 α) and of a rabbit polyclonal antibody to this protein were generously provided by Dr. U. Walter (Medizinische Universitätsklinik, Würzburg, Germany). Cilostamide was a gift from Professor H. Hidaka (Nagoya University, Nagoya, Japan). Zaprinast (M&B 22948) was from May & Baker (Dagenham, Essex, U.K.) and milrinone from Sterling Drug Inc. (Rensselaer, NY, U.S.A.).

Preparation of platelet cytosol and membranes

Human platelets were isolated and resuspended in Ca²⁺-free Tyrode's solution (37 °C) containing 5 mM Pipes (buffered to pH 6.5 with NaOH), 50 units of heparin/ml and 75 μ g of apyrase/ml by modification of the method of Mustard et al. [15]. After centrifugation (1000 *g* for 15 min), the platelets were finally resuspended at 2.5×10^9 /ml in a solution containing 100 mM KCl, 25 mM Hepes (buffered to pH 7.4 with NaOH), 2.5 mM EGTA and 0.2 mM leupeptin (solution A). The suspension was

Abbreviations used: cGMP, cAMP, cyclic GMP and cyclic AMP respectively; PDE III, cGMP-inhibited cAMP phosphodiesterase; RI and RII, regulatory subunits of cAMP-dependent protein kinase; cGMP-PK, cGMP-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; Rp-8-bromo-cGMPs, 8-bromoguanosine cyclic 3',5'-monophosphorothioate, Rp isomer; Sp-cAMPS, adenosine cyclic 3',5'-monophosphorothioate, Sp isomer; Rp-cAMPS, adenosine cyclic 3',5'-monophosphorothioate, Rp isomer; PDE II, cGMP-stimulated cyclic nucleotide phosphodiesterase; PDE V, cGMP-binding cGMP phosphodiesterase.

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then frozen in methanol/solid CO₂ and stored at -70 °C. Platelets were then lysed by freezing and thawing twice, and the lysate was centrifuged at 40000 *g* for 40 min to isolate the membrane fraction, which was resuspended in solution A. The protein concentrations in the supernatant (cytosol) and membrane fractions were determined [16] and adjusted to 1 mg/ml.

Photoaffinity labelling of platelet proteins by [³²P]cGMP

Incubation mixtures (100 µl) containing 75–80 µl of platelet cytosol or membrane suspension and 20–25 µl of additions were placed in the wells of a Microtest III 96-well tissue-culture plate (Falcon 3072) resting in an ice tray. Additions were dissolved in dimethyl sulphoxide (final concn. 0.2%, v/v) or in solution A and included 5 µCi of [³²P]cGMP (30–60 nM after dilution) and dithiothreitol (1 mM after dilution). Nickel-wire stir bars were added to each well. After 15 min equilibration in the dark, the stirred samples were irradiated for a further 15 min with a 30 W germicidal lamp (Westco, Hamilton, ON, Canada) placed 5 cm above the open plate. The irradiated samples received 1.1 mW/cm² (J-225 u.v. meter; Ultraviolet Products Inc., San Gabriel, CA, U.S.A.). This experimental design permitted simultaneous labelling of multiple samples in a reproducible manner. A 90 µl portion of each sample was then mixed with 0.5 ml of 10% (w/v) trichloroacetic acid. After 30 min, the precipitated protein was isolated by centrifugation, dissolved in electrophoresis sample buffer and analysed by SDS/PAGE (usually with 10% acrylamide) [17]. Dried gels were exposed overnight on Kodak X-OMAT AR film.

Preparation of anti-PDE III antiserum

A synthetic peptide corresponding to the C-terminal 14 residues of human platelet PDE III [18] was synthesized and conjugated to rabbit serum albumin by Multiple Peptide Systems (San Diego, CA, U.S.A.). Male New Zealand White rabbits were given multiple intradermal injections of peptide conjugate (1 mg of peptide/rabbit) mixed with Freund's complete adjuvant. After four booster injections of conjugate mixed with Freund's incomplete adjuvant, the rabbits were bled out and sera were evaluated by immunoblotting of platelet proteins; a serum that at 1:1000 dilution gave a 110 kDa signal that was blocked by 10 µM immunizing peptide was selected.

Immunoprecipitation

Samples (100 µl) containing photoaffinity-labelled proteins were diluted to 1 ml with a solution containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1% (v/v) Nonidet P40 and 0.5% (w/v) sodium deoxycholate (solution B) supplemented with 1 mM phenylmethanesulphonyl fluoride, 0.25 mM phenanthroline and 0.2 mM leupeptin. Any insoluble proteins were removed by centrifugation at 100000 *g* for 30 min. Both the cytosolic and soluble membrane proteins were pre-cleared twice with heat-killed formaldehyde-fixed *Staphylococcus aureus* (i.e. rocked for 2 h at 4 °C). Each of the labelled protein fractions was then incubated for 1 h at 0 °C with 5 µl of antiserum, followed by agitation for 2 h at 4 °C with 50 µl of 50% Protein A-Sepharose. The Protein A-Sepharose was isolated by centrifugation (12000 *g*, 1 min), washed three times with solution B, resuspended in electrophoresis sample buffer [17] and boiled for 5 min before analysis by SDS/PAGE.

RESULTS

Factors affecting photoaffinity labelling of platelet proteins by [³²P]cGMP

Preincubation and u.v. irradiation of a mixture of platelet cytosol fraction and [³²P]cGMP (see the Experimental section) led to a selective incorporation of ³²P into 110, 55, 49 and 38 kDa proteins, as well as to less marked labelling of a few other proteins (100, 85 and 80 kDa) (Figure 1a). Both the 49 kDa and 38 kDa proteins appeared as closely spaced doublets in briefly exposed autoradiographs. Irradiation was essential for the labelling of all species, except the cytosolic 38 kDa proteins (Figure 1a, lane 1). In platelet membrane fractions, less ³²P was incorporated into the 110 kDa protein and much more into the 80 kDa species (Figure 1b). In addition, photolabelled membranes contained a 65 kDa labelled protein not found in cytosol, a 49 kDa protein corresponding to that in the cytosol (but no 55 kDa species) and a 38 kDa protein that co-electrophoresed with the upper cytosolic component.

Addition of MgCl₂ (5 mM) exerted differential effects on the labelling of these proteins, enhancing that of most species, but diminishing that of the 80 kDa protein (Figure 1). In view of possible losses of [³²P]cGMP by enzymic hydrolysis, we studied the effects of zaprinast, an inhibitor of cGMP phosphodiesterase [19]. At 10 µM, this compound enhanced photolabelling of some of the above proteins in both the presence and absence of MgCl₂, and particularly that of the 110 kDa species (Figure 1). Both MgCl₂ and zaprinast were therefore used in the subsequent experiments that focused on the cytosolic 110 kDa protein, whereas zaprinast alone was used in experiments on platelet membranes and the 80 kDa protein. In the presence of these additions, increases in the amounts of cytosolic protein (10–80 µg) and of [³²P]cGMP (1–8 µCi) were associated with corresponding increases in ³²P incorporation into the above-

Figure 1 Effects of Mg²⁺ and zaprinast on the photoaffinity labelling of human platelet cytosolic and membrane proteins by [³²P]cGMP

Samples containing platelet cytosol (lanes 1–5) and membranes (lanes 6–10) were incubated with [³²P]cGMP at 0 °C. Protein from lanes 1 and 6 was not irradiated, whereas that in lanes 2–5 and 7–10 was irradiated for 15 min at 0 °C. Other additions were: lanes 1, 2, 6 and 7, none; lanes 3 and 8, 5 mM MgCl₂; lanes 4 and 9, 10 µM zaprinast; lanes 5 and 10, 5 mM MgCl₂ and 10 µM zaprinast. Protein was analysed by SDS/PAGE; an autoradiograph is shown. The positions of standard proteins (see the text) are shown on the right (◀) and the calculated molecular masses (*M*) of labelled proteins on the left.

Figure 2 Effects of various compounds on the photoaffinity labelling of human platelet cytosolic proteins by [^{32}P]cGMP

All samples contained 5 mM MgCl_2 and 10 μM zaprinast. Other conditions (all 10 μM) were: lane 1, none; lane 2, cGMP; lane 3, cAMP; lane 4, cilostamide; lane 5, milrinone; lane 6, IBMX; lane 7, GMP. After irradiation, protein from each sample was analysed by SDS/PAGE (13% acrylamide); an autoradiograph is shown. The calculated molecular masses (M) of the labelled proteins are shown on the left.

Figure 3 Effects of various compounds on the photoaffinity labelling of human platelet membrane proteins by [^{32}P]cGMP

All samples contained 10 μM zaprinast, but no MgCl_2 . Other additions (all 20 μM) were: lane 1, none; lane 2, cGMP; lane 3, 8-bromo-cGMP; lane 4, Rp-8-bromo-cGMP; lane 5, GMP; lane 6, cAMP; lane 7, Sp-cAMPS; lane 8, Rp-cAMPS; lane 9, AMP. After irradiation, protein from each sample was analysed by SDS/PAGE; an autoradiograph is shown. The calculated molecular masses (M) of the labelled proteins are indicated on the left.

labelled species. We used 80 μg of protein and 5 μCi of [^{32}P]cGMP in most experiments, as this gave strong signals after autoradiography overnight.

Effects of competing compounds on photoaffinity labelling by [^{32}P]cGMP

We compared the effects of 10 μM concentrations of various compounds on the photolabelling of platelet cytosolic proteins by [^{32}P]cGMP (Figure 2). Unlabelled cGMP decreased the labelling of all the proteins studied, but particularly that of the 110 kDa species. However, cAMP only inhibited labelling of the latter protein and, unexpectedly, induced marked labelling of the 80 kDa species. Photolabelling of the 110 kDa protein was partially inhibited by IBMX and potently inhibited by the specific PDE III inhibitors, cilostamide and milrinone [20], which

Figure 4 Immunoprecipitation of photoaffinity-labelled platelet proteins using antisera to platelet PDE III and to bovine lung cGMP-PK

(a) Human platelet cytosol was photoaffinity-labelled with [^{32}P]cGMP in the presence of 5 mM MgCl_2 and 5 μM zaprinast. Control labelled protein (33 μg) and immunoprecipitates from 80 μg of labelled protein were analysed by SDS/PAGE; an autoradiograph is shown. Lane 1, control; lane 2, immunoprecipitate using anti-PDE III antiserum; lane 3, immunoprecipitate using anti-PDE III antiserum plus 5 μM immunizing peptide; lane 4, immunoprecipitate using preimmune serum. (b) human platelet membranes were photoaffinity labelled with [^{32}P]cGMP in the presence of 10 μM zaprinast alone (lanes 1 and 2) or of 10 μM zaprinast and 10 μM cAMP (lanes 3–6). Control labelled protein (53 μg) and immunoprecipitates from 80 μg of membrane protein were analysed by SDS/PAGE; an autoradiograph is shown. Lanes 1 and 3, controls; lanes 2 and 4, immunoprecipitates using anti-cGMP-PK antiserum; lane 5, immunoprecipitate using anti-cGMP-PK antiserum plus cGMP-PK (0.8 μg); lane 6, immunoprecipitate using non-immune serum. The positions of standard proteins and the calculated molecular masses (M) of the labelled proteins are shown.

had only weak effects on the labelling of other proteins. GMP had no effect on labelling of the 110 kDa protein, but did to some extent inhibit labelling of the 55, 49 and 38 kDa species. AMP (20 μM) had no significant effects (results not shown).

In similar experiments with platelet membranes (Figure 3), 20 μM concentrations of cGMP, 8-bromo-cGMP and Rp-8-bromo-cGMPs inhibited photolabelling of the 80, 65, 49 and 38 kDa proteins, whereas the same concentration of GMP had no such effects. At 20 μM , either cAMP or Sp-cAMPS markedly increased labelling of the 80 kDa protein, though these two compounds inhibited labelling of all other proteins (Figure 3). An additional 76 kDa species, presumably related to the 80 kDa protein, was usually detected when labelling of the latter was highly stimulated by cAMP or Sp-cAMPS. In contrast with Sp-cAMPS, 20 μM Rp-cAMPS decreased labelling of the 80 kDa protein, in addition to inhibiting labelling of the 65, 49 and 38 kDa species. AMP (20 μM) had no effects on the photolabelling of any of these membrane proteins.

Identification of the 110 kDa and 80 kDa proteins

By using a rabbit antiserum to PDE III, we were able to immunoprecipitate the 110 kDa photolabelled protein from platelet cytosol (Figure 4a). The only other labelled proteins in the immunoprecipitate were the minor 100 kDa and 85 kDa species. Addition of immunizing peptide prevented immunoprecipitation of labelled proteins, and none were seen when preimmune serum was used. We also selectively immunoprecipitated the 80 kDa photolabelled protein from detergent-solubilized platelet membrane fraction, using an antiserum to bovine lung cGMP-PK (Figure 4b). When cAMP was added to enhance photolabelling, the 76 kDa species was immunoprecipitated with the 80 kDa protein. Immunoprecipitation of

Figure 5 Photoaffinity labelling of purified bovine lung cGMP-PK by [32 P]cGMP: effects of platelet membranes and cAMP

Samples containing cGMP-PK (0.4 μ g) (lanes 1 and 6), membranes (75 μ g of protein) (lanes 2 and 4) or both cGMP-PK and membranes (lanes 3 and 5) were photoaffinity-labelled in the presence of 10 μ M zaprinast (lanes 1–3) or of 10 μ M zaprinast and 10 μ M cAMP (lanes 4–6). The labelled protein was analysed by SDS/PAGE; an autoradiograph is shown. The positions of standard proteins are shown on the right and the calculated molecular masses (M) of the labelled proteins on the left. The 32 P in the 80 kDa labelled protein was determined (407, 88, 1321, 288, 2114 and 132 d.p.m. in lanes 1–6 respectively).

these proteins was decreased by purified bovine lung cGMP-PK and was not seen when non-immune serum was used.

To confirm that the 80 kDa labelled protein was platelet cGMP-PK, we photolabelled a sample of the purified 76 kDa type I α enzyme from bovine lung and obtained a labelled species that migrated at 80 kDa on SDS/PAGE (Figure 5, lane 1). In the absence of membranes, cAMP inhibited photoaffinity labelling of purified cGMP-PK (Figure 5, lane 6). However, photolabelling of the enzyme was greatly enhanced by the addition of membranes in the absence or presence of cAMP (Figure 5, lanes 3 and 5). The total photolabelling obtained with a mixture of 75 μ g of membrane protein and 0.4 μ g of purified cGMP-PK was 2.5–5-fold greater than the sum of that seen when these components were labelled individually.

DISCUSSION

Human platelets contain a cGMP-inhibited cAMP phosphodiesterase (PDE III) with an apparent subunit molecular mass on SDS/PAGE of 110 kDa [20]. Two lines of evidence indicate that the 110 kDa protein photolabelled by [32 P]cGMP corresponds to this enzyme. First, photoaffinity labelling of this species was blocked not only by cGMP and cAMP, but also by specific PDE III inhibitors, including cilostamide and milrinone. Second, the labelled protein was selectively immunoprecipitated by an antibody to PDE III. Although an early study showed that PDE III could be photolabelled by [32 P]cGMP in K30a lymphoma cells, which overexpress this enzyme [10], PDE III has not previously been detected by this method in platelets. This enzyme is readily proteolysed [20], and it is likely that the minor 85 and 100 kDa photolabelled species found in platelet cytosol, which were also immunoprecipitated by PDE III antiserum, are proteolytic fragments of this enzyme. It is also possible that PDE III and these fragments conceal some cGMP-stimulated phosphodiesterase (PDE II), which has an apparent molecular mass of 102–105 kDa [19] and is present in small amounts in platelets [21]. The 84–99 kDa cGMP-specific phosphodiesterase (PDE V) [19], which is a major cGMP-binding species in platelets

[22], was not observed in our experiments, despite a preliminary report that it can be photolabelled in platelets [11]. This discrepancy was not attributable to the addition of zaprinast. Also, we have shown (K. M. Tang, J. L. Sherwood and R. J. Haslam, unpublished work) that it is not due to the omission of IBMX, which potentiates the binding of cGMP to this enzyme [22]. However, a higher pH than was used in the present experiments is optimal for cGMP binding to platelet PDE V [22,23].

Several lines of evidence indicate that the 80 kDa photolabelled protein is the platelet cGMP-PK [2]. First, these proteins display the same subcellular distribution. Secondly, the 80 kDa labelled protein co-migrated with the purified photolabelled bovine lung (type I α) enzyme. Thirdly, antibody to the bovine lung cGMP-PK immunoprecipitated the 80 kDa photolabelled protein. Finally, cGMP analogues that bind to cGMP-PK, including its 8-bromo- and 3',5'-monophosphorothioate derivatives [24,25], inhibited photolabelling. In contrast, cAMP and Sp-cAMPS greatly stimulated labelling of the 80 kDa protein, though the Rp-cAMPS diastereomer was inhibitory. A similar effect of cAMP on the photolabelling of a platelet membrane protein was briefly reported by Walseth et al. [11], although the labelled species was not identified. Since cAMP did not stimulate photolabelling of the purified bovine lung enzyme, and both diastereomers of cAMPS have been shown to inhibit binding of cGMP to this protein [26], it seemed possible that autophosphorylation or cAMP-dependent phosphorylation of the membrane cGMP-PK might be responsible for the effect observed in membranes. Although a slight decrease in the electrophoretic mobility of cGMP-PK photolabelled in the presence of cAMP (Figure 5) is consistent with this possibility, no other evidence for phosphorylation of this enzyme was obtained in preliminary experiments. Indeed, autophosphorylation of cGMP-PK has been reported to enhance cAMP binding [27], which should decrease photolabelling. Addition of platelet membranes to purified cGMP-PK with or without cAMP greatly facilitated photolabelling of this enzyme. Although the factors in platelet membranes responsible for these effects have not been identified, our results indicate that cAMP may be able to increase cGMP binding to platelet cGMP-PK, and suggest the possibility of a novel co-operative interaction between the effects of these cyclic nucleotides.

The 65 kDa platelet membrane protein that was photoaffinity-labelled does not correspond to any known platelet cGMP-binding protein. This protein is unlikely to be a proteolytic fragment of cGMP-PK, since it was not immunoprecipitated with this enzyme and its labelling was affected differently by Mg^{2+} . Since photolabelling of the 65 kDa protein was inhibited by both cyclic nucleotides, it is possible that it represents a novel membrane site of action of these compounds. It may be relevant that the apparent molecular mass of the subunit of the retinal rod cGMP-gated ion channel is 63 kDa and that similar cyclic-nucleotide-gated ion channels exist in other cell types [6,7,28].

It is likely that the 49 and 55 kDa platelet proteins photolabelled by [32 P]cGMP are the RI and RII regulatory subunits of cAMP-dependent protein kinase, which migrate more slowly on SDS/PAGE than expected from their true molecular masses [29]. Thus, these photolabelled proteins had molecular masses and subcellular distributions identical with those previously observed after photoaffinity labelling of platelet RI and RII by 8-azido-cAMP [30]. Photolabelling of RI by [32 P]cGMP has also been observed in K30a lymphoma cells. The 38 kDa group of labelled proteins may be proteolytic fragments of the RI and/or RII regulatory subunits, since species of this size were also observed after photoaffinity labelling with 8-azido-cAMP and were increased in amount after Ca^{2+} -dependent proteolysis [30].

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