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α_{2A}-Adrenergic receptors activate protein kinase C in human platelets via a pertussis toxin-sensitive G-protein

Rienk Nieuwland, Odilia L.C. Wijburg, Gijsbert van Willigen, Jan-Willem N. Akkerman*

Department of Haematology, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands

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Abstract

4,4′-Diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) stimulates human platelets via α_{2A}-adrenergic receptor-mediated activation of protein kinase C (PKC) independent of the phospholipase C pathway. Here we show, that in permeabilized platelets activation of PKC by DIDS (20 μM), measured as ^{32}P incorporation in pleckstrin, is completely inhibited by guanosine 5′-(2-*O*-thio)diphosphate (200 μM), an inhibitor of heterotrimeric G-proteins. Also pertussin toxin (4 μg/ml), which ADP-ribosylates the α-subunits of G_i's and G_o, prevents pleckstrin phosphorylation by DIDS. *N*-Ethylmaleimide (50 μM), which uncouples G_i from α_{2A}-adrenoceptors, inhibits pleckstrin phosphorylation by DIDS in intact platelets. Activation of PKC by 55 nM phorbol 12-myristate 13-acetate and 500 nM platelet-activating factor are not disturbed by NEM. DIDS inhibits by 40 ± 5% (n = 4) the pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of a 41 kDa protein fraction previously shown to contain the α-subunits of $G_i\alpha$-1, $G_i\alpha$-2 and $G_i\alpha$-3. Thus, the α_{2A}-adrenergic receptor activates PKC via a G-protein of the G_i-family.

Key words: α_2-Adrenergic receptor; GTP-binding protein; Protein kinase C; Platelet

1. Introduction

Agonists such as α-thrombin, ADP and platelet-activating factor (PAF) activate human platelets via phospholipase C (PLC)-dependent signal transduction. PLC cleaves phosphatidylinositol 4,5-biphosphate in inositol 1,4,5-triphosphate (IP_3) and *sn* 1,2-diacylglycerol (DG), which respectively release intracellular Ca^{2+} ions [1] and activate protein kinase C (PKC) [2,3]. A second activating pathway is mediated via phospholipase A_2 (PLA_2) and formation of prostaglandin endoperoxides/thromboxane A_2, which induce platelet functions via a secondary, PLC-mediated mechanism [4,5]. Subsequent reactions lead to the exposure of fibrinogen binding sites on the glycoprotein (GP) IIB/IIIA complex, aggregation and secretion of granule contents.

Epinephrine induces platelet aggregation via activation of PLA_2 independent of the PLC pathway, provided that secondary activation by prostaglandin endoperoxides/thromboxane A_2 is prevented [4–6]. Despite the absence of DG formation there is evidence for increased PKC activity, since the PKC inhibitor staurosporine prevents GP IIB/IIIA exposure by epinephrine [7–9]. Also phosphorylation of the PKC substrate pleckstrin is enhanced by epinephrine independent of formation of DG or phosphatidic acid [8,9]. These findings suggest, that the α_{2A}-adrenergic receptor (α_{2A}-AR) activates PKC via a mechanism that does not depend on DG formation.

Sage et al. [10] showed, that 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS, 100 μM), an inhibitor of Cl^-/HCO_3^- exchange [11,12], increased the cytosolic pH in platelets, although platelets lack a Cl^-/HCO_3^- exchanger. Previously Periasamy et al. [13] that DIDS interacts with the α_{2A}-AR on human platelets. Recently we described that at much lower concentrations (2–20 μM) of DIDS also enhance Na^+/H^+ exchange, but also activates PKC and induces fibrinogen binding to GP IIB/IIIA all via a mechanism that was inhibited by the α_{2A}-AR antagonists oxymetazoline and yohimbine [14]. These responses were initiated without accumulation of phosphatidic acid, cytosolic Ca^{2+} and cyclooxygenase products, suggesting that PLC and PLA_2 were not involved. In contrast, the PKC inhibitor staurosporine inhibited Na^+/H^+ exchange, exposure of GP IIB/IIIA and aggregation, indicating that activation of PKC was an early step in signal generation by the [DIDS]–[α_{2A}-AR] complex.

Many receptors trigger signal processing via heterotrimeric G-proteins [15,16]. In the present study we investigated whether G-proteins formed a link between the α_{2A}-AR and PKC during platelet activation by DIDS.

* Corresponding author. Fax: (31) (30) 511 893.
E-mail: haematologie@pobox.ruu.nl

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2. Materials and methods

2.1. Materials

Staurosporine, guanosine-5'-*O*-(2-thiophosphate) GDP(β)S), nicotinamide adenine dinucleotide (NAD) and PAF were derived from Boehringer (Mannheim, Germany) and Sepharose 2B from Pharmacia (Uppsala, Sweden). Alpha-thrombin, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), phorbol-12-myristate-13-acetate (PMA), *N*-ethylmaleimide (NEM), ATP, indomethacine, thymidine and pertussis toxin were from Sigma (St. Louis, MO, USA). [^{32}P]Orthophosphate (spec.act. 8500–9120 Ci/mmol) and [^{32}P]nicotinamide adenine dinucleotide di(triethylammonium)salt ([^{32}P]NAD; spec.act. 800 Ci/mmol) were derived from New England Nuclear (Boston, MA, USA). Fibrinogen (grade L) was obtained from KABI (Stockholm, Sweden) and saponin from BDH Chemicals LTD (Poole, UK). All other chemicals were of analytical grade.

2.2. Blood collection

Freshly drawn venous blood from healthy volunteers was collected (with informed consent) into tri-sodium citrate (0.1 vol. of 130 mM). The donors claimed not to have taken any medication during the previous ten days.

2.3 Measurement of PKC activity

Citrated blood was centrifuged (200 × *g*, 10 min, 22°C) and the platelet rich plasma (PRP) was collected and acidified to pH 6.5 with 1/6 vol of ACD (2.5 g tri-sodium citrate, 1.5 g citric acid, 2.0 g D-glucose in 100 ml water). Platelets were labelled with 0.1 mCi [^{32}P]orthophosphate/ml acidified PRP (pH 6.5) in the presence of indomethacine (30 μM) for 1 h at 37°C. The platelets were isolated by centrifugation (700 × *g*, 15 min, 22°C) and resuspended in HEPES-Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES pH 7.2), resulting in a final platelet count of (150–200) × $10^3/\mu$l. Labelled platelets were stimulated at 37°C. In several experiments permeabilized platelets were used. After centrifugation, the ^{32}P-loaded platelets were resuspended in a buffer containing 160 mM KCl, 2.3 mM $MgCl_2$, 12 mM HEPES, 10 mM thymidine, 0.5 mM ATP, 0.5 mM NAD and pertussis toxin (4 μg/ml). Platelets were permeabilized by saponin (20 μg/ml) added 2 min before addition of agonists. Samples were collected at the times indicated in section 3 and transferred into 0.5 vol of 3× concentrated Laemmli sample buffer and heated for 2 min at 100°C prior to electrophoresis. Proteins were separated by electrophoresis through a 11% polyacrylamide gel, according to Laemmli [17]. Gels were stained with Coomassie brilliant blue and the distribution of radioactivity was determined by autoradiography of dried gels on Kodak Royal X-Omat film. For determination of the radioactivity of pleckstrin, the specific area was cut out of the dried gels and heated for 2 h at 80°C in 30% H_2O_2. The radioactivity was determined by liquid scintillation counting. Data are expressed as percentage of the maximal phosphorylation of pleckstrin induced by 1 min stimulation with α-thrombin (1 U/ml) to minimize donor variation.

2.4 [^{32}P]ADP ribosylation by pertussis toxin

PRP was acidified with ACD to pH 6.5 and platelets were isolated by centrifugation (700 × *g*, 15 min, 20°C). The platelet pellet was resuspended in HEPES-Tyrode buffer (pH 7.2), to a final platelet count of 800 × $10^3/\mu$l. Platelets were stimulated for 5 min at 30°C before the addition of 3 vols. of ribosylation buffer, containing pertussis toxin (13 μg/ml), [^{32}P]NAD (67 μM), saponin (20 μg/ml), 160 mM KCl, 27 mM HEPES, 5 mM $MgCl_2$ and 13 μM EGTA. After 1 h incubation at 30°C, the permeabilized platelets were collected by centrifugation (12,000 × *g*, 30 s, 22°C) and solubilized in three times concentrated Laemmli sample buffer and heated for 5 min at 100°C. Proteins were separated by electrophoresis through a 11% polyacrylamide gel and the radioactivity in the 41 kDa protein was determined, as described in section 2.3. Data are expressed as percentage inhibition of the amount of [^{32}P]ADP ribosylation in unstimulated platelets.

2.5. Presentation of data

Data are expressed as means ± S.D., unless indicated otherwise. Statistical significances were determined by Student's *t*-test and were considered significant at $P < 0.05$. All experiments were performed with 3–5 different platelet preparations.

3. Results

3.1. Effect of GDP(β)S on DIDS-induced pleckstrin phosphorylation

Addition of DIDS (20 μM, final con.) to platelets induced a 4- to 5-fold increase in ^{32}P-labelled pleckstrin, illustrating activation of PKC (Fig. 1). The α_{2A}-receptor antagonist oxymetazoline [18] abolished the increase in [^{32}P]pleckstrin completely, indicating that DIDS activated PKC via the α_{2A}-AR. A similar inhibition of PKC activation by DIDS was obtained following a 2 min preincubation with GDP(β)S (200 μM), provided that the platelets were made permeable with saponin [19]. Control studies confirmed that in the absence of platelet activators saponin did not change pleckstrin phosphorylation (data not shown). On the other hand, a combina-

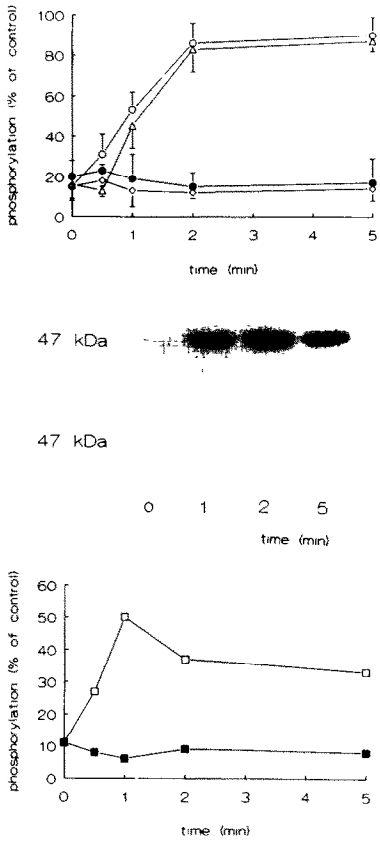
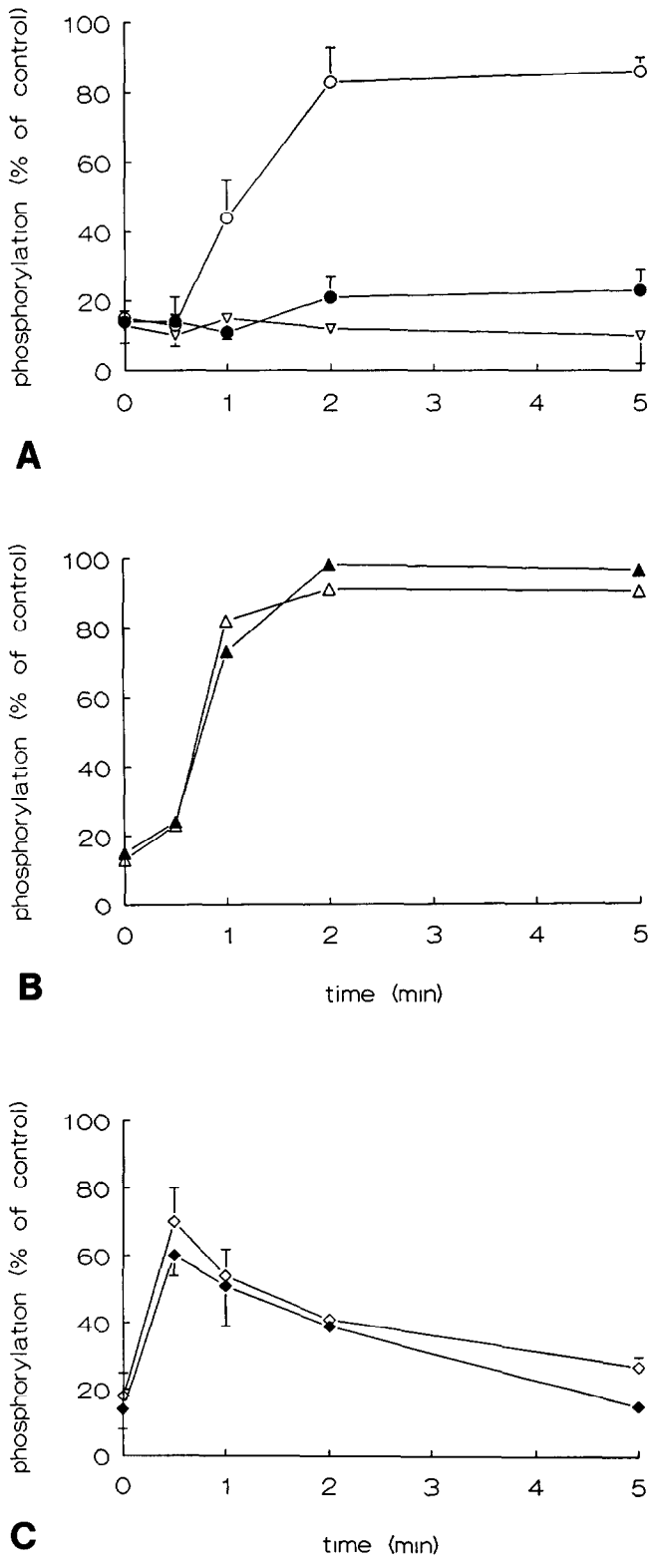


Fig. 1. Effect of GDP(β)S on DIDS-induced pleckstrin phosphorylation. The upper figure shows ^{32}P-labeled platelets pre-incubated with buffer (△), 500 μM oxymetazoline (◇), 200 μM GDP(β)S (○) or GDP(β)S in combination with 20 μM saponin (●) for 2 min at 37°C. Subsequently, platelets were stimulated with DIDS (20 μM). The radioactivity in pleckstrin was measured at different times after stimulation. The phosphorylation data are expressed as percentage of the maximal α-thrombin (1 U/ml)-induced increase in pleckstrin phosphorylation. The 47 kDa spots show the DIDS-induced increase in pleckstrin phosphorylation in the presence of GDP(β)S (upper panel) or GDP(β)S in the presence of saponin (lower panel) at different times after addition of agonist. The lower figure shows pleckstrin phosphorylation after stimulation with α-thrombin (0.1 U/ml) in the presence of GDP(β)S (200 μM) with (■) or without (□) preincubation with saponin (20 μg/ml).

tion of saponin and GDP(β)S also prevented the increase in [^{32}P] pleckstrin by α-thrombin (0.1 U/ml), confirming earlier observations that thrombin activates PKC via a G-protein-dependent pathway [20]. These findings indicate that the activation of PKC of DIDS depends on G-proteins.



3.2. *Effect of NEM on DIDS-induced pleckstrin phosphorylation*

NEM alkylates cysteine residues of the α-subunit of G_i and uncouples $G_i\alpha$-2 from the α_{2A}-AR [15,21–24]. Fig. 2A shows that the increase in [^{32}P]pleckstrin induced by DIDS was completely inhibited after a 1 min preincubation with 50 μM NEM. Unstimulated platelets showed no change in ^{32}P-radioactivity in pleckstrin during treatment with NEM (data not shown). To investigate which step in the activation by DIDS was sensitive to NEM, the experiments were repeated during direct stimulation of PKC. As shown in Fig. 2B, NEM did not affect pleckstrin phosphorylation induced by 55 nM PMA, indicating that the NEM sensitive step seen during stimulation by DIDS was not at the level of PKC activation. Furthermore, the NEM sensitivity appeared specific for α_{2A}-AR-mediated activation of PKC, since phosphorylation of pleckstrin induced by 500 nM PAF (Fig. 2C) and 1 U/ml α-thrombin (data not shown) was not sensitive to NEM treatment. Together these data suggest, that one or more G-proteins of the G_i-family take part in the activation of PKC by the [α_{2A}-AR]–[DIDS] complex.

A second means to demonstrate the involvement of G-proteins of the G_i-family is by analyzing ADP-ribosylation by pertussis toxin [24]. This toxin catalyzes the transfer of an ADP-ribose moiety, derived from NAD^+, to a cysteine-residue of the α-subunit of G_i [25], thereby blocking signal transduction. As also shown in Fig. 2A, preincubation of saponin-treated platelets with pertussis toxin inhibited pleckstrin phosphorylation by DIDS completely, illustrating that activation of PKC by DIDS is mediated by a G-protein of the G_i-class.

3.3. *[^{32}P]ADP ribosylation in platelets treated with DIDS*

To demonstrate the pertussis toxin sensitivity of DIDS-induced PKC activation in more detail, we determined the effect of DIDS on [^{32}P]ADP ribosylation of α_i. Pertussis toxin ribosylates the unstimulated, intact heterotrimeric G-protein, whereas the liberated α_{GDP} subunit is no longer susceptible to ADP-ribosylation [22,24]. As shown in Fig. 3 (lane 1), [^{32}P]NAD-labelled platelets showed a considerable incorporation of the

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Fig. 2. Effect of NEM and pertussis toxin on pleckstrin phosphorylation induced by DIDS. (A) ^{32}P-labeled platelets preincubated with buffer (○) or with 50 μM NEM (●) for 2 min at 37°C. Subsequently, platelets were stimulated with DIDS (20 μM). Alternatively, ^{32}P-labeled platelets were preincubated with saponin (20 μg/ml) and pertussis toxin (4 μg/ml) for 2 min at 37°C before addition of 20 μM DIDS (▽). The radioactivity in pleckstrin was measured at different times after stimulation. The phosphorylation data are expressed as percentage of maximal α-thrombin (1 U/ml) induced increase in pleckstrin phosphorylation. (B) ^{32}P-labeled platelets stimulated with PMA (55 nM) without (△) or after a 2-min preincubation with 50 μM NEM (▲). (C) ^{32}P- labeled platelets stimulated with PAF (500 nM) without (◇) or after a 2-min preincubation with 50 μM NEM (◆).

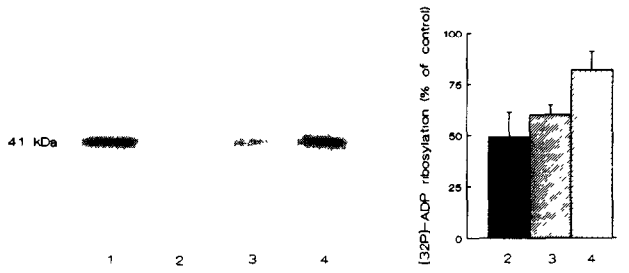

Fig. 3. Effect of DIDS on pertussis toxin catalyzed [^{32}P]ADP-ribosylation. Platelets ($800 \times 10^3/\mu l$) were stimulated with α-thrombin (1 U/ml) or DIDS (20 μM) for 5 min at 30°C. The stimulated platelets were diluted in 3 vols. of ribosylation buffer, containing pertussis toxin (13 μg/ml), saponin (20 μg/ml) and [^{32}P]NAD (67 μM). Following a 1-h incubation at 30°C, the [^{32}P]ADP-ribosylation was measured. The left panel shows a representative autoradiogram of unstimulated platelets (lane 1) and platelets stimulated with α-thrombin (1 U/ml; lane 2), DIDS (20 μM; lane 3) or epinephrine (10 μM; lane 4). The right panel summarizes the data from 3 different experiments. The data are expressed as percentage inhibition of the [^{32}P]ADP-ribosylation measured in unstimulated platelets (means ± S.D.; $n = 3$).

^{32}P-label in a 41 kDa protein, previously shown to contain different forms of α_i [26]. Addition of α-thrombin (1 U/ml) reduced the ADP-ribosylation by 51 ± 12% ($n = 3$; lane 2). Addition of DIDS (20 μM) reduced the ADP-ribosylation by 40 ± 5% ($n = 3$; lane 3). Also epinephrine, the natural agonist for the α_{2A}-adrenergic receptor, inhibited ADP-ribosylation, although less potently than DIDS (lane 4). These data indicates, that DIDS activates a G protein, which is ADP-ribosylated by pertussis toxin.

4. Discussion

In a previous study we showed that DIDS activates platelets via the α_{2A}-AR [14]. This activation was mediated by PKC and resulted in activation of the Na^+/H^+ exchanger, exposure of fibrinogen binding sites on the GP IIb/IIIa complex and aggregation. The present study indicates that activation of PKC by the [α_{2A}-AR]–[DIDS] complex depends on one or more G-proteins of the G_i family. Evidence for this concept comes from the observation that the phosphorylation of pleckstrin is inhibited by GDP(β)S, NEM, and pertussis toxin, and that DIDS inhibits [^{32}P]ADP ribosylation of a 41 kDa protein consisting of the α_i subtypes 1, 2 and 3 [26].

Human platelets are a prototypic tissue for the α_{2A}-AR. The receptor contains seven transmembrane spanning domains characteristic for G protein-coupled receptors [18]. The α_{2A}-AR is coupled to $G_i\alpha$-2, which is sensitive to pertussis-toxin [21,26]. Antibodies directed against the C-terminal part of this G-protein blocked the α_{2A}-AR-induced inhibition of adenylyl cyclase, indicating that activation of $G_i\alpha$-2 is a crucial step in this pathway [15]. The α_{2A}-AR expressed in LLC-PK1-O-cells couples not only to $G_i\alpha$-2 but also to $G_i\alpha$-1 and $G_i\alpha$-3 [27]. Both $G_i\alpha$-a and $G_i\alpha$-3 are present in platelets [26], but at present it is unknown whether these G_i's are activated by the α_{2A}-AR. When in fibroblasts both $G_i\alpha$-2 and $G_i\alpha$-3 were activated via the α_{2A}-AR, only $G_i\alpha$-2 mediated inhibition of adenylyl cyclase and it is possible that also in platelets $G_i\alpha$-2 is more important than the other subtypes [28].

The α-subunits of $G_i\alpha$-1, $G_i\alpha$-2 and $G_i\alpha$-3 are highly conserved and have more than 90% amino acid sequence homology [29]. One of the conserved amino acids is a C-terminal cysteine located at position 351 or 352, which is a substrate for pertussis toxin [22,24,29]. The S1 subunit of this toxin is an ADP-ribosyltransferase, that catalyzes the transfer the ADP-ribose moiety of NAD^+ to this C-terminal cysteine [25]. The same cystein is alkylated by NEM [22,24], which disrupts its interaction with pertussis toxin [22]. Modification of this cysteine uncouples the G-protein from the activated receptor, abolishes signal generation and lowers the affinity of the receptor for its agonists. The affinity of the α_{2A}-AR for [^{3}H]UK 14,304 was about 30% lower after 30 min incubation with 100 μM NEM [30].

Both the inhibition of AC [31,32] and activation of PKC (this study) via the α_{2A}-AR are abolished by pertussis toxin. A similar inhibition is seen with NEM ([23], this study), leading to complete blockade of α_{2A}-AR-induced GTPase activity [33]. In platelets activated via the thrombin receptor a different mechanism appears to be involved. Again the inhibition of AC [33] and activation of PKC [31,32] are abolished by pertussis toxin but NEM only prevents the inhibition of AC ([33], this study). In line with this observation is the 30% inhibition by NEM of α-thrombin induced GTPase activity [33]. By analogy with the α_{2A}-AR, the thrombin receptor may inhibit AC via $G_i\alpha$-2. This α-subtype contains a second cysteine at position 112. Alkylation of this cysteine left the GTP-binding site intact [22], but prevented reassociation of the α-subunit with the β-complex [24]. The activation of PKC by α-thrombin must then be mediated via another G-protein of the G_i-family with a lower sensitivity to NEM. $G_i\alpha$-1 and $G_i\alpha$-3 might be less sensitive to NEM since they lack the extra cysteine of $G_i\alpha$-2.

Recently, Pan et al. [34] showed activation of a phosphotyrosine phosphatase from a human pancreatic cell line by a pertussis-toxin sensitive G-protein. Our present findings indicate that a serine/threonine kinase activity is regulated by G-proteins. Thus, phosphorylation (this study) as well as dephosphorylation [34] may be under direct control of G-proteins.

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