Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1776–1780, March 1989
Biochemistry

Epinephrine induces changes in the subcellular distribution of the inhibitory GTP-binding protein $G_{i\alpha\text{-}2}$ and a 38-kDa phosphorylated protein in the human platelet

(thrombin/platelet activation/membrane skeleton)

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Communicated by Pedro Cuatrecasas, December 2, 1988 (received for review September 2, 1988)

ABSTRACT **By using antibodies specific for α subunits of inhibitory GTP-binding proteins ($G_{i\alpha}$ polypeptides) to probe Western blots of whole platelet protein, we detected $G_{i\alpha\text{-}2}$ as the predominant $G_{i\alpha}$ species present in platelets. The subcellular compartmentalization of distinct $G_{i\alpha\text{-}2}$-immunoreactive polypeptides coupled to thrombin and α_2-adrenergic receptors was examined in Triton X-100 platelet lysates prepared by high-speed centrifugation. This treatment permitted separation of the Triton-insoluble membrane skeleton from Triton-soluble cell components. In cells treated with either α-thrombin or epinephrine, we observed that a greater proportion of $G_{i\alpha\text{-}2}$ was localized in the Triton-soluble fraction than in the Triton-insoluble fraction. Pertussis toxin was found to catalyze ADP-ribosylation of $G_{i\alpha\text{-}2}$ in whole platelets. In thrombin-stimulated cells, this activity was confined to the Triton-soluble fraction and was markedly lower than that of unstimulated cells. Epinephrine, on the other hand, promoted translocation of a portion of the pertussis toxin-sensitive $G_{i\alpha\text{-}2}$ from the Triton-soluble fraction to the Triton-insoluble fraction. In addition, epinephrine stimulated translocation of a phosphorylated protein of ≈38 kDa that was not ADP-ribosylated by pertussis toxin. This protein expressed immunoreactivity with the general $G_{i\alpha}$ antiserum AS/7 but not with the $G_{i\alpha\text{-}2}$ antiserum LE/3. These findings suggest a role for specific localization of $G_{i\alpha}$ proteins in epinephrine-induced platelet responses.**

Among platelet agonists, epinephrine is unique in that its action on α_2-adrenergic receptors induces aggregation independent of the biochemical events usually associated with platelet activation (1, 2). For other agonists, stimulation of platelet function is associated with the turnover of inositol phospholipids, mobilization of intracellular calcium stores, and activation of protein kinase C (3). None of these events is seen in direct response to epinephrine (1).

When added in combination with other agonists, subthreshold concentrations of epinephrine potentiate the responsiveness of these agonists to phospholipase C activation (2, 4). Our previous studies have indicated that this effect resides in an ability of α_2-adrenergic receptors to enhance the coupling of, for instance, the thrombin receptor to phospholipase C. The mechanism of this effect is largely unknown; however, we previously suggested that the effects of epinephrine may be mediated by G_i, the inhibitory GTP-binding protein (2).

A number of subtypes of G_i have been described recently. These have been characterized in brain and neutrophils by immunoreactivity with antibodies raised against synthetic peptides representing portions of the α subunit of G_i ($G_{i\alpha}$) as predicted by their cDNA sequences (5–8). We have used these antibodies to show that the predominant species in platelets is $G_{i\alpha\text{-}2}$.

We found that epinephrine, but not thrombin, stimulated the translocation of $G_{i\alpha\text{-}2}$ and a 38-kDa potential $G_{i\alpha}$-like protein from a Triton-soluble fraction of the cell to a Triton-insoluble, membrane skeleton compartment. We suggest that the association of $G_{i\alpha\text{-}2}$ proteins with the membrane skeleton may be involved in the aggregatory action of epinephrine and therefore may represent a novel pathway of activating platelet responses.

EXPERIMENTAL PROCEDURES

Materials. α-Thrombin was a generous gift of John Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany). Antisera to $G_{i\alpha}$ and $G_{o\alpha}$ were kindly supplied by Allen Spiegel (National Institutes of Health) and the G_i/G_o standard by Peter Gierschik (Department of Pharmacology, University of Heidelberg, Heidelberg). The $G_{\alpha,common}$ antiserum was provided by Susanne Mumby and Alfred Gilman (University of Texas Health Center, Dallas). Epinephrine, yohimbine, and phorbol 12,13-dibutyrate were purchased from Sigma; carrier-free [^{32}P]-orthophosphoric acid was from ICN; [^{32}P]NAD (1000 Ci/mmol; 1 Ci = 37 GBq) from DuPont/New England Nuclear; and pertussis toxin from List Biochemicals (Campbell, CA).

Preparation of ^{32}P-Labeled Platelets. Platelets from 100 ml of blood were isolated as described (2) and resuspended in 5 ml of buffer (138 mM NaCl/2.9 mM KCl/20 mM Hepes, pH 7.4/1 mM glucose/1 mM $MgCl_2$). [^{32}P]Orthophosphate (5 mCi) was then added and the cells were left at 37°C for 90 min. Cells were then diluted to 25 ml, centrifuged, and resuspended in buffer containing 3.3 mM NaH_2PO_4, 1 mM EGTA, and 1 mM aspirin. After 20 min the cells were again pelleted and resuspended in 5 ml of buffer without EGTA or aspirin. During periods of labeling or centrifugation, prostacyclin (100 ng/ml) was added to inhibit activation.

Preparation of Triton X-100-Soluble and -Insoluble Platelet Fractions. Aliquots (0.5 ml) of platelets in buffer were incubated with various agonists for the indicated times, and a sample (75 μl) was taken and added to an equal volume of Triton X-100 lysis buffer [62.5 mM Tris·HCl/0.223 mM Tris base/10% (vol/vol) glycerol/2% (vol/vol) Triton X-100/5 mM EGTA, pH 7.4, with phenylmethylsulfonyl fluoride at 0.2 mg/ml]. Triton X-100-soluble and -insoluble fractions were separated by centrifugation for 30 min at 170,000 × g in an air-driven ultracentrifuge (Beckman Airfuge). The resulting supernatant (Triton-soluble fraction) was diluted with 150 μl of SDS-containing sample buffer [60 mM Tris, pH 7.4/10% (vol/vol) glycerol/3% (wt/vol) SDS/3% (wt/vol) dithiothrei-

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Abbreviations: G_i, inhibitory GTP-binding protein; $G_{i\alpha}$, α subunit of G_i.

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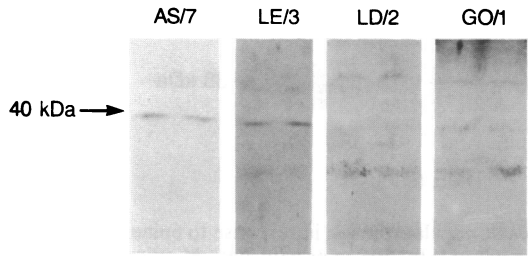

FIG. 1. Binding of antibodies directed against all $G_{i\alpha}$ subunits or against $G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$, or $G_{o\alpha}$ to Western blots of whole platelet protein. Aliquots of human platelets were added to sample buffer, and the proteins were separated by SDS/PAGE and then electrophoretically transferred to nitrocellulose paper. Strips of the paper blotted from the same gel were incubated with antiserum AS/7 (recognizes all $G_{i\alpha}$ subunits; dilution 1:2000), LE/3 (recognizes $G_{i\alpha\text{-}2}$; dilution 1:100), LD/2 (recognizes $G_{i\alpha\text{-}1}$; dilution 1:100), or GO/1 (recognizes $G_{o\alpha}$; dilution 1:100). Reactive proteins were visualized by using an alkaline phosphatase-linked second antibody. In each panel, the two lanes represent duplicate loadings of identical sample preparations.

tol]. The pellet (Triton-insoluble fraction) was rinsed with 50 μl of 0.5× Triton X-100 lysis buffer and recentrifuged for 5 min. The resulting pellet was resuspended in 50 μl of 0.5× Triton X-100 lysis buffer plus 50 μl of SDS-containing sample buffer. Both fractions were heated to 100°C for 4 min prior to separation of the proteins (50 μl from each fraction) by SDS/11% PAGE (9). Some gels were Western blotted (i.e., the proteins were electrophoretically transferred to nitrocellulose) and the blots were probed for $G_{i\alpha}$-immunoreactive proteins as described (2). ^{32}P-labeled proteins were visualized by autoradiography.

Measurement of Pertussis Toxin-Induced ADP-Ribosylation in Permeabilized Platelets. Platelets from 50 ml of blood were incubated at 37°C for 20 min in buffer containing EGTA and aspirin (1 mM each), centrifuged for 10 min, and resuspended in buffer without EGTA (2 ml). Platelet samples (50 μl) were added to Airfuge tubes containing the appropriate agonist and 30 sec later, the ADP-ribosylation mixture was added (15 μl). The ADP-ribosylation mixture contained saponin (20 μg/ml), nonradioactive NAD (20 μM), [*adenylate*-^{32}P]NAD (2 μCi/ml), pertussis toxin (7 μg/ml, preactivated with 1.1 mM dithiothreitol for 30 min at 37°C), EDTA (1 mM), thymidine (10 mM), GTP (17 μM), and diethylenetriaminepentaacetic acid (2 μM). Following a 20-min incubation, the reaction was halted by the addition of Triton X-100 lysis buffer (75 μl). Triton-soluble and -insoluble platelet fractions were then prepared as described above.

RESULTS

Whole platelet protein that had been Western-blotted to nitrocellulose bound $G_{i\alpha}$ antibody AS/7 (which reacts with both $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$; ref. 6) and $G_{i\alpha\text{-}2}$-specific antibody LE/3 (7). No reactivity was found with $G_{i\alpha\text{-}1}$-specific antibody LD/2 (7) or an antibody directed against $G_{o\alpha}$ (6) (Fig. 1).

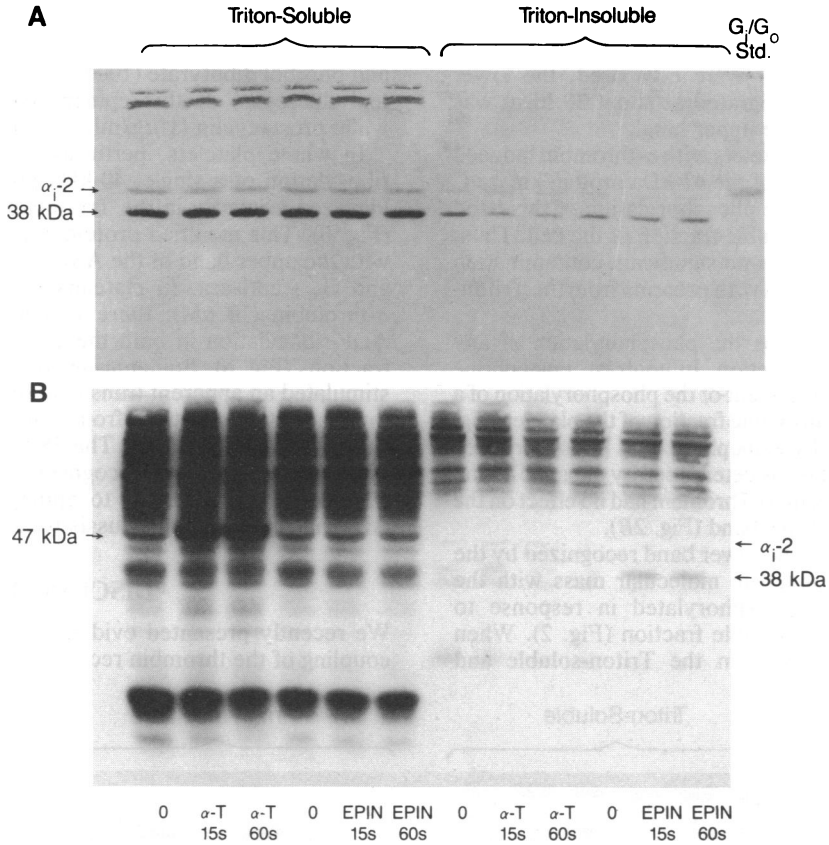

FIG. 2. (*A*) Western blots of whole platelet protein probed for $G_{i\alpha}$ with AS/7 antiserum. Platelets were activated with or without α-thrombin (α-T, 10 nM) or epinephrine (EPIN, 100 μM) for 15 or 60 sec, an equal volume of Triton X-100 lysis buffer was added, and Triton-soluble and -insoluble fractions were separated by high-speed centrifugation. SDS-containing sample buffer was added to both fractions, and the proteins were separated by SDS/11% PAGE and blotted to nitrocellulose. The blots were probed with the $G_{i\alpha}$ antiserum AS/7, and immunoreactive proteins were visualized with an alkaline phosphatase-linked second antibody. Results are representative of eight different preparations. (*B*) Translocation of a phosphorylated 38-kDa protein to the Triton-insoluble fraction of platelets in response to epinephrine. ^{32}P-labeled platelets were prepared and treated as described for *A*. Shown is an autoradiograph of the Western blot presented in *A*. The lower molecular mass protein band recognized by the AS/7 antiserum corresponded in molecular mass with the band that increased in radioactivity in the Triton-insoluble fraction in response to epinephrine, as indicated.

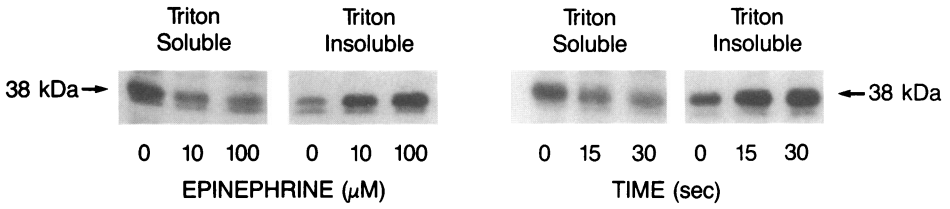

FIG. 3. Translocation of the 38-kDa protein from Triton-soluble to Triton-insoluble cellular fraction in response to epinephrine. ^{32}P-labeled platelets were prepared and treated with various amounts of epinephrine (0, 10, or 100 μM) for 30 sec or with 100 μM epinephrine for various times (0, 30, or 60 sec). Triton-soluble and Triton-insoluble fractions were prepared from the activated platelets as described for Fig. 2*A*. Shown are autoradiographs of platelet proteins separated by SDS/11% PAGE. Note the concentration- and time-dependent increases in the amount of 38-kDa protein translocated from the Triton-soluble fraction to the Triton-insoluble fraction in response to epinephrine.

To examine the distribution of $G_{i\alpha}$ among subcellular compartments involved in the regulation of platelet shape changes, samples were treated with 1% Triton X-100 and the Triton-insoluble membrane skeleton was isolated by high-speed centrifugation (10). Using $G_{i\alpha}$-specific antibodies, we detected $G_{i\alpha\text{-}2}$ in both Triton-soluble and Triton-insoluble fractions, although a greater proportion was localized in the Triton-soluble fraction (Fig. 2*A*). The immunoreactivity of these samples with a new preparation of the general $G_{i\alpha}$ antiserum AS/7 (Fig. 2*A*) was different from that observed previously (Fig. 1). This antiserum preparation recognized two distinct protein bands on immunoblots of both Triton-treated samples and whole platelet protein. The upper band separated on SDS/PAGE just below that of a brain $G_{i\alpha}$ preparation that presented one immunoreactive band at 41 kDa. In addition, the position of the upper band corresponded exactly with the single band identified by $G_{\alpha,common}$ (A-569) and $G_{i\alpha\text{-}2}$ antisera. When the relative intensities of immunoreactivities with AS/7 were compared, the lower band from platelets, which migrated at about 38 kDa, was much more prevalent than the upper band.

Activation of ^{32}P-labeled platelets with α-thrombin induced the classical phosphorylation of the 47-kDa protein kinase C substrate (Fig. 2*B*). The entire phosphorylation of this band was confined to the Triton-soluble fraction of the cell. Thus, we are confident that there was no significant contamination of the Triton-insoluble samples with proteins from the Triton-soluble fraction.

Epinephrine did not increase the phosphorylation of any bands in the Triton-soluble fraction. In contrast, epinephrine produced an apparent enhancement of the phosphorylation of a protein band in the Triton-insoluble fraction of the platelet (Fig. 2*B*). The protein affected by epinephrine was phosphorylated exclusively on serine residues as determined by phospho amino acid analysis (data not shown). α-Thrombin had no effect on the level of phosphorylation of this band (Fig. 2*B*).

The immunoreactivity of the lower band recognized by the AS/7 antiserum corresponded in molecular mass with the band (38 kDa) that was phosphorylated in response to epinephrine in the Triton-insoluble fraction (Fig. 2). When the radioactivity of this band in the Triton-soluble and Triton-insoluble fractions was determined by scintillation counting, we found that the epinephrine-induced increase in radioactivity of the 38-kDa band in the Triton-insoluble fraction was mirrored by a decrease in radioactivity of this band in the Triton-soluble fraction (Fig. 2*B*). There was no difference in the radioactivity of this band between control and epinephrine-stimulated cells that did not undergo lysis with Triton X-100-containing buffer. This suggested that, rather than stimulating phosphorylation of the 38-kDa protein, epinephrine was acting to promote its translocation from a Triton-soluble fraction of the cell to a Triton-insoluble compartment. The amount of 38-kDa protein translocated to the Triton-insoluble fraction was greater at increasing concentrations of epinephrine and longer periods of incubation with epinephrine (Fig. 3).

The ability of epinephrine (100 μM) to translocate this band was reduced by yohimbine (50 μM), an α_2-adrenergic-selective antagonist (Figs. 4 and 5). Both α-thrombin (10 nM) and phorbol dibutyrate (100 nM) produced slight but consistent decreases in this epinephrine-induced translocation, while prostacyclin (1 μg/ml) had no effect (Fig. 5).

In whole platelets, pertussis toxin catalyzed the ADP-ribosylation of a single, 40-kDa protein (2, 11), which was localized primarily in the Triton-soluble fraction of the cell (Fig. 6). This modified protein expressed immunoreactivity with the upper band of the AS/7 doublet and with $G_{\alpha,common}$ and $G_{i\alpha\text{-}2}$ antisera. In platelets stimulated for 30 sec with α-thrombin (10 nM), there was marked inhibition of this ADP-ribosylation in both the Triton-soluble and -insoluble fractions (Fig. 6). Epinephrine (100 μM), on the other hand, stimulated an apparent translocation of the 40-kDa pertussis toxin-sensitive substrate from Triton-soluble fraction to the Triton-insoluble fraction. The 38-kDa protein, which comigrates with a protein recognized by AS/7 and which is translocated in response to epinephrine, did not undergo ADP-ribosylation by pertussis toxin.

DISCUSSION

We recently presented evidence that G_i is involved in the coupling of the thrombin receptor to phospholipase C in the

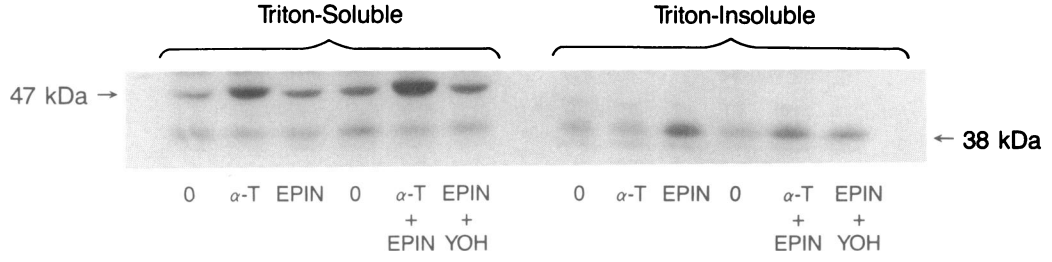

FIG. 4. Translocation of the 38-kDa protein by epinephrine: Effect of α-thrombin and yohimbine. ^{32}P-labeled platelets were prepared and treated with α-thrombin (α-T, 10 nM, 30 sec) and/or epinephrine (EPIN, 100 μM, 30 sec) or with epinephrine and yohimbine (YOH, 50 μM, 60 sec). Triton-soluble and -insoluble fractions were prepared as in Fig. 2*A*. Shown is an autoradiograph of platelet proteins separated by SDS/PAGE. Note the epinephrine-induced changes in the amount of phosphorylated 38-kDa protein in the Triton-insoluble fraction.

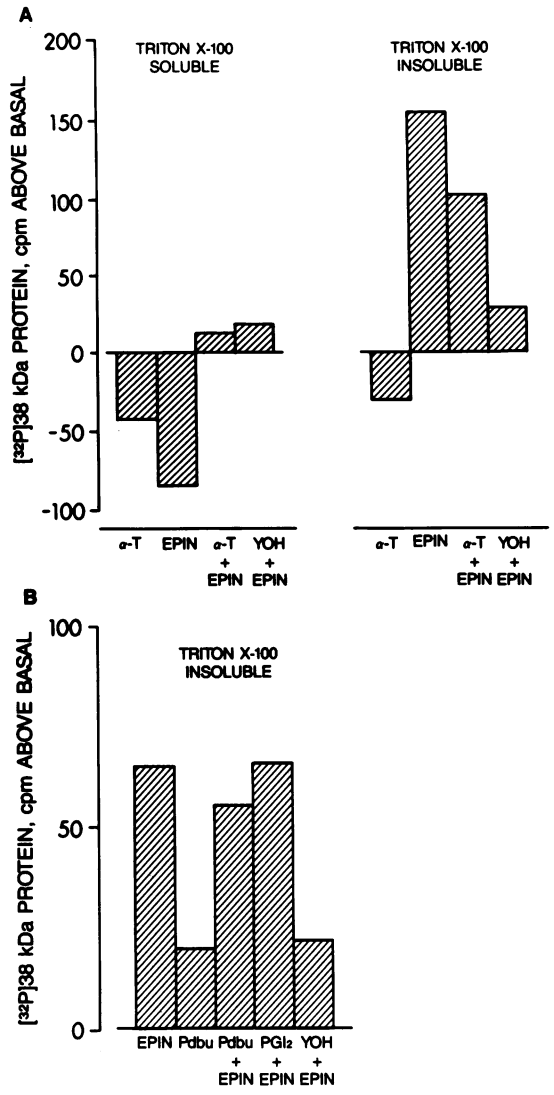

FIG. 5. Translocation of the 38-kDa protein from Triton-soluble to Triton-insoluble cellular fractions in response to epinephrine. The band corresponding to the 38-kDa phosphorylated protein was cut from the SDS/PAGE gel for measurement of radioactivity in a scintillation counter. The counts were corrected for the percentage of each sample loaded onto the gel. The basal level of radioactivity was 591 cpm in the Triton-soluble fraction and 211 cpm in the Triton-insoluble fraction. Results are representative of three experiments. (α-T, α-thrombin, 10 nM, 30 sec; EPIN, epinephrine, 100 μM, 30 sec; YOH, yohimbine, 50 μM, 60 sec.) (*B*) Translocation of the 38-kDa protein by epinephrine: effect of phorbol ester, prostacyclin, and yohimbine. The effect of epinephrine on translocation of the 38-kDa phosphorylated protein was examined as in *A*, but only the radioactivity of the band in the Triton-insoluble fraction is presented. The basal level of radioactivity of the Triton-insoluble fraction was 137 cpm. These results are representative of two separate experiments. (EPIN, epinephrine, 100 μM, 30 sec; PdBu, phorbol 12,13-dibutyrate, 100 nM, 30 sec; PGI_2, prostacyclin, 1 μg/ml, 60 sec; YOH, yohimbine, 50 μM, 60 sec.)

human platelet (2). Both the thrombin receptor and the α_2-adrenergic receptor are known to be linked to G_i, but the α_2-adrenergic receptor does not stimulate phospholipase C. Because of this, we felt that the role of G_i was to modulate the specific effects of these receptors and that another GTP-binding protein was actually responsible for the direct coupling of the thrombin receptor to phospholipase C.

Using specific antisera against $G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$, and $G_{o\alpha}$, we found strong reactivity with an antibody directed against $G_{i\alpha\text{-}2}$, but were unable to detect $G_{i\alpha\text{-}1}$ or $G_{o\alpha}$ on Western blots

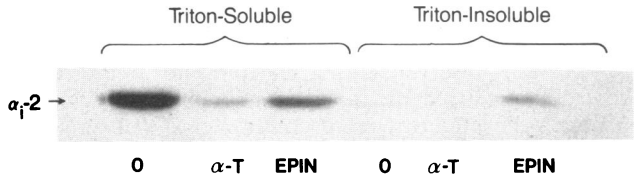

FIG. 6. Pertussis toxin-induced ADP-ribosylation of $G_{i\alpha\text{-}2}$ in Triton-solubilized platelets. Human platelets were treated with aspirin, resuspended in buffer, and incubated with no addition (0), α-thrombin (α-T, 10 nM), or epinephrine (EPIN, 100 μM) for 30 sec. After this time, an ADP-ribosylation mixture was added that included saponin, [^{32}P]NAD, and pertussis toxin. The reaction was quenched after 20 min by the addition of Triton X-100 lysis buffer. Samples were fractionated into Triton-soluble and Triton-insoluble compartments as described for Fig. 2. The autoradiograph of labeled proteins separated by SDS/PAGE is presented. Results are representative of three different preparations.

of whole platelet protein or immunoprecipitated $G_{i\alpha}$ (data not shown). We do not yet have evidence for or against the existence of $G_{i\alpha\text{-}3}$ in platelets, but based on the strong immunoreaction with the $G_{i\alpha\text{-}2}$ antiserum, we feel that it is likely that this is the major species of $G_{i\alpha}$ in these cells.

Thus far, the structural and functional characterization of GTP-binding proteins in platelets has not included a thorough examination of the subcellular organization of these proteins in stimulated and unstimulated cells. It is known that platelets undergo dramatic morphological changes upon exposure to certain agonists (10). These changes are accompanied by aggregation and secretion and are thought to be linked to the activation of cytoskeleton and membrane skeleton-associated contractile mechanisms within the cell. To determine whether these agonist-induced platelet shape changes are in some way coupled to receptors via $G_{i\alpha\text{-}2}$, we looked for changes in G_i-protein content in the membrane skeleton upon cell activation by thrombin or epinephrine.

We detected only a small amount of $G_{i\alpha\text{-}2}$ associated with the high-speed Triton-insoluble fraction of the platelet in both control cells and in cells stimulated with either thrombin or epinephrine. Western blots of Triton X-100-treated cells revealed that most of the $G_{i\alpha\text{-}2}$ protein was present in the Triton-soluble fraction. $G_{i\alpha\text{-}2}$ was ADP-ribosylated by pertussis toxin in both cellular compartments; however, a much greater proportion of the modified protein was found in the Triton-soluble fraction. α-Thrombin inhibited the ADP-ribosylation of $G_{i\alpha\text{-}2}$, whereas epinephrine promoted translocation of a portion of the ADP-ribosylated protein to the Triton-insoluble fraction.

We have shown here that our preparations of platelet protein presented two immunoreactive bands with the AS/7 antiserum: the $G_{i\alpha\text{-}2}$ band at 40 kDa and a second, lower molecular mass protein band. The lower band appeared to migrate at the same position as a 38-kDa phosphorylated protein that was translocated from the Triton-soluble fraction of the cell to the Triton-insoluble fraction in response to epinephrine. This evidence suggests that the 38-kDa protein shares amino acid sequence homology with a specific region of $G_{i\alpha\text{-}2}$ and may be a $G_{i\alpha}$-like protein. However, we were unable to demonstrate its immunoreactivity with other $G_{i\alpha}$-specific antisera or its ADP-ribosylation by pertussis toxin. It is possible that the 38-kDa protein represents an altered form of $G_{i\alpha\text{-}2}$ that has retained an intact carboxyl-terminal antibody-recognition site but that no longer assumes the appropriate conformation for ADP-ribosylation by pertussis toxin.

Translocation of the pertussis toxin-sensitive $G_{i\alpha\text{-}2}$ to the Triton-insoluble fraction of the platelet in response to epinephrine suggests a potential interaction between a G_i protein and cellular components involved in the regulation of platelet shape changes—i.e., the platelet membrane skeleton. This interaction may represent a novel way of stimulating platelet

aggregation that does not involve the mobilization of calcium stores or the activation of phospholipase C, responses that are normally observed during platelet activation. Hence, aggregation induced by the interaction of $G_{i\alpha\text{-}2}$ with the membrane skeleton may represent a novel way of activating platelet responses.

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