Subcellular distribution of α_2 -adrenergic receptors, pertussis-toxin substrate and adenylate cyclase in human platelets

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The subcellular distribution of the α_2 -adrenergic receptor, pertussis-toxin substrates (G_i , the inhibitory G-protein) and adenylate cyclase was determined in human platelets. The α_2 -adrenergic receptor and pertussis-toxin substrate activity codistribute with surface membranes identified by a novel fluorescent-lectin method. The platelet granule fractions did not contain detectable G_i . Only 2–4 % of the total pertussis-toxin substrate activity appears in soluble fractions, and this amount was not increased upon addition of purified $\beta\gamma$ units or after pretreatment of platelets with adrenaline. There is no evidence for compartmentation of the α_2 -adrenergic receptor or G_i to account for the low-affinity component of agonist binding to the α_2 -adrenergic receptor in human platelet membranes. Translocation of G_i from plasma membrane to platelet cytosol or granules does not appear to play any significant role in the mechanism of α_2 -receptor-mediated platelet activation.

INTRODUCTION

Regulation of many cellular functions by drugs and hormones involves membrane receptors and guanine-nucleotide-binding proteins (G-proteins) (see ref. [1] for review). In addition to mediating stimulation and inhibition of adenylate cyclase, other effector responses have been shown to be mediated by G-proteins, including stimulation of phospholipases A_2 and C [2–4] and regulation of ion channels [5].

It has been proposed that the α subunit of G-proteins can be released from the plasma membrane upon receptor activation [6]. Experimental support for the existence of soluble α subunit is well established for the rhodopsin-transducin system [7]. Similar suggestions have been made for G-proteins in neutrophils [8], liver membranes [9], S49 lymphoma-cell membranes [10] and human platelets [11,12]. The movement of G-protein α subunits to intracellular locations other than the plasma membrane has significant implications for the types of processes that can be activated by those transducing proteins. Specifically, cytosolic or secretory granule mechanisms could be activated. Conversely, loss of α subunits from the plasma membrane would result in uncoupling of receptors from their plasma membrane responses. Many reports of such soluble G-protein subunits are only qualitative, and do not give a reliable estimate of the fraction of the G-protein that is soluble. A quantitative account of the distribution of G-protein subunits is necessary to place in perspective the hypotheses based on the presence of soluble α subunits.

Human blood platelets have been a useful system for studies of the mechanism of the G_i -mediated adenylate cyclase inhibition induced by α_2 -adrenergic agonists [13–15]. Both ligand binding to G-protein-linked receptors and functional desensitization may depend on the relative distribution of the receptor and its transducing G-protein. Agonist binding to α_2 -adrenergic receptors in platelet membranes is characterized by two affinity states [15–17]. The high-affinity α_2 -agonist binding requires a pertussis-toxin-sensitive G-protein [18,19] and appears to represent the functional form of the α_2 receptor [20]. In addition to the high-affinity binding, α_2 agonists bind approx. 30–40 % of the platelet α_2 receptors with low affinity. These ‘low-affinity’ receptors appear to be unable to couple to G_i , either because the receptor itself is heterogeneous or because compartmentation of the receptor or G_i prevents them from interacting with each other [15,21]. Indeed, desensitization of the β -adrenergic receptor is accompanied by sequestration of the receptor in a membrane compartment that is devoid of functional G_s [22,23].

We report here determination of the pertussis-toxin substrate (G_i) in various subcellular fractions of the human platelet and compare its distribution with that of the α_2 -adrenergic receptor and adenylate cyclase. Three questions are addressed: (1) what is the quantitative amount of G_i in the soluble fraction and in the various membrane fractions? (2) are the α_2 receptor and G_i distributed in the same membrane compartments? and (3) does desensitization of the platelet α_2 -adrenergic response modify the distribution of receptor or G_i in human platelets?

Abbreviations used: FITC, fluorescein isothiocyanate; G-protein, GTP-binding regulatory protein; G_s , stimulatory G-protein; G_i , inhibitory G-protein; PRP, platelet-rich plasma.

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MATERIALS AND METHODS

Sources of materials

[³H]Yohimbine (70–90 Ci/mmol), [4,5-³H]UK 14304 (70–90 Ci/mmol), [³²P]NAD⁺ (10–50 Ci/mmol), [U-¹⁴C]sucrose (671 mCi/mmol) and [¹⁴C]tryptamine bisuccinate (43.4 mCi/mmol) were obtained from New England Nuclear. [α -³²P]ATP (10–30 Ci/mmol) and cyclic [³H]AMP (30–50 Ci/mmol) were obtained from Amersham. Purified pertussis toxin was purchased from List Biologicals or was kindly provided by Dr. Larry Winberry of the State of Michigan Department of Public Health. Forskolin was obtained from Calbiochem-Behring. The following reagents were obtained from Sigma: (–)-adrenaline (+)-bitartrate, cytochrome *c* (type III), antimycin A, tryptamine (free base), NADH (disodium salt), phenylmethanesulphonyl fluoride, Triton X-100, Lubrol PX and fluorescein isothiocyanate (FITC)-labelled *Lens culinaris* lectin (2–4 mol of FITC/mol of protein). All other chemicals were obtained from standard commercial sources and were of reagent grade or better.

Collection and preparation of platelets

Human platelet concentrates were purchased from the Detroit Chapter of the American Red Cross within 36 h of collection ('stored platelets'). Platelets were isolated essentially as described by Neubig & Szamraj [24], except that 0.25 M-sorbitol was used instead of sucrose in the freezing and homogenization buffer to permit lectin labelling. In some experiments platelets from freshly drawn blood were used and were prepared as follows. Blood from normal aspirin-free donors was collected by venous puncture after an overnight fast. Processing followed immediately at room temperature. The blood was mixed with $\frac{1}{5}$ vol. of ACD anticoagulant (0.8 % citric acid, H₂O, 2.2 % trisodium citrate, 2H₂O, 2.45 % dextrose) and centrifuged at 280 *g*_{max} for 7 min. Platelet-rich plasma (PRP) was collected and its pH adjusted to 6.50 with ACD to inhibit aggregation. Platelets were pelleted from the PRP by centrifugation at 420 *g*_{max} for 15 min and then resuspended in an equal volume of Tris-buffered saline (20 mM-Tris/HCl, 150 mM-NaCl, 1 mM-Na₂EDTA, pH 7.6). The platelets were pelleted as above and resuspended in buffer containing 10 mM-Tris/HCl, 5 mM-Na₂EDTA and 0.25 M-sorbitol, pH 7.6 at 4 °C (homogenization buffer), before being frozen in a solid-CO₂/ethanol bath for storage at –70 °C for less than 1 week before use. Fractionation of platelets obtained from the Red Cross and those obtained from freshly drawn blood yielded slightly different results (see below). All experiments involving freshly drawn platelets were conducted by following protocols approved by the Human Subjects Committee of the University of Michigan.

Surface labelling of intact platelets with FITC-labelled *Lens culinaris* lectin

In some experiments, the intact platelets were labelled with fluorescent lectin. After being pelleted from the PRP, the platelets were resuspended in $\frac{1}{10}$ vol. of Tris-buffered saline. FITC-labelled *Lens culinaris* lectin reconstituted at a concentration of 2 mg/ml in 0.01 M-sodium phosphate/150 mM-NaCl, pH 7.4, supplemented with 0.02 % NaN₃ and 0.1 mM each of MgCl₂, MnCl₂ and CaCl₂ was added to the platelets, yielding a final

concentration of 25 µg/ml. The suspension was incubated at 20 °C for 20 min. No visible agglutination was noted. The platelets were then pelleted, washed, resuspended, and stored as above. Pretreatment with lectin under these conditions did not alter the distribution of [³H]yohimbine binding, G_i, adenylate cyclase, monoamine oxidase, antimycin A-insensitive NADH-dependent cytochrome *c* reductase, lactate dehydrogenase, or protein (results not shown).

Pretreatment of platelets with adrenaline

PRP was prepared from freshly drawn blood as described above. Aspirin (100 µM) was added and the mixture incubated at room temperature for 10 min to prevent subsequent aggregation. The aspirin-treated platelets were then divided into two parts. To one half 100 µM-(–)-adrenaline (+)-bitartrate and 0.005 % sodium ascorbate was added, and to the other half 0.005 % sodium ascorbate alone was added. These mixtures were incubated for 3 h at room temperature, at which time the pH was adjusted to 6.50 with ACD, followed by processing as described above.

Homogenization and subcellular fractionation of platelets

Immediately after thawing the platelets in a water bath at 23 °C, 100 µM-phenylmethanesulphonyl fluoride was added from a 100 mM stock in ethanol. Platelets were homogenized in 3.5 ml portions (each representing approx. 1.5 unit of platelet concentrate or the platelets obtained from 200 ml of fresh blood) for 8 min in a 'zero-clearance' homogenizer (Kontes no. 866030-023) with a Talboys model 106 motor set at 30 % ($\approx$ 250 rev./min). Homogenization was performed on ice in a cold-room, and was done for a constant period of time rather than a constant number of strokes, as the former was found to yield more consistent results. Under these conditions, 70–95 % of the total lactate dehydrogenase was found in soluble fractions (results not shown). Except where noted, the homogenate was then centrifuged at 1200 *g*_{max} for 5 min at 4 °C to remove undisrupted and partially disrupted platelets. Samples of the crude homogenate and the low-speed supernatant were diluted 5-fold with 30 % (w/w) sorbitol, and the low-speed pellet was resuspended in 1 ml of the same. These samples were included in all assays for the purpose of calculating yields and recoveries.

A 2 ml portion of the supernatant from the low-speed spin was layered on 16 ml linear 30–60 % sorbitol gradients. The gradients were centrifuged at 130 000 *g*_{max} in a Beckman SW27.1 rotor for 3 h at 4 °C. Fractions were collected from the top of the gradients with an Isco gradient collector by pumping 65 % sorbitol in the bottom. This method of collection yielded superior resolution of peaks as compared with collection from the bottom. The fractions were diluted 10-fold with a buffer containing 50 mM-Tris/HCl and 1 mM-EGTA, pH 7.6, and then pelleted by centrifugation at 145 000 *g*_{max} for 30 min at 4 °C, followed by resuspension in 1.0–0.3 ml of the same buffer. When larger quantities of membrane fractions were required, platelets were homogenized in 3 ml portions, followed by fractionation as above on two to four parallel gradients. Fractions were collected as above and pooled on the basis of visually estimated turbidity into the three major particulate fractions (L, M and H; see the Results section).

To quantify soluble G_i , platelet homogenates were centrifuged at 48 000 g for 20 min, and then the supernatant was removed and centrifuged a second time for 100 000 g to eliminate any possible contamination by membranes. Portions of the pellet and high-speed supernatant were assayed for G_i activity as described below.

Partially purified human platelet plasma membranes

These were prepared by the method of Neubig & Szamraj [24].

Purified α_o and $\beta\gamma$ subunits of bovine brain G proteins

These were prepared as described in ref. [19].

Assays

All assays were performed within 4 days after homogenization on fractions stored at 4 °C.

α_2 -Adrenergic receptor. Binding of the α_2 -adrenergic antagonist [3H]yohimbine was measured as previously described [15]. Determination of the α_2 receptor in gradient samples used 10 nM-[3H]yohimbine, a concentration approximately twice the K_d . Control experiments with pooled membrane fractions (L, M and H) showed that the differences in the amount of [3H]yohimbine binding were due to differences in the B_{max} , rather than the K_d for the antagonist. In some experiments, binding of 1 nM-[3H]UK 14304, an α_2 -selective agonist, was also measured as described in ref. [15].

G_i . Pertussis-toxin-catalysed [^{32}P]ADP-ribosylation in cholate extracts of membrane fractions was performed by the method of Bokoch *et al.* [25] as modified [15]. The cholate extracts diluted in Lubrol were quick-frozen in a solid- CO_2 /ethanol bath and stored at -70 °C before being assayed. Incubation of extracts with pertussis toxin (10 $\mu g/ml$) and [^{32}P]NAD $^+$ (2 μM) were carried out as previously described [15]. ^{32}P incorporation into protein was quantified by autoradiography of dried gels followed by scintillation counting of the labelled region for radioactivity or by densitometry of autoradiograms. In some experiments, purified G-protein $\beta\gamma$ subunits were added to the incubations (see below).

Adenylate cyclase. Adenylate cyclase activity was measured as previously described [15] on pelleted fractions. Assays were performed immediately after fractionation to avoid loss of activity. Where noted, 10 μM -forskolin was included in the assay. In experiments assessing latency of the enzyme, the membrane fractions were pretreated with the non-ionic detergent Lubrol PX by incubation on ice in the presence of Lubrol PX for 1 h, followed by 1:1 dilution into the standard assay cocktail.

FITC-lectin. FITC fluorescence of gradient fractions was measured by mixing the samples with an equal volume of 2% SDS, followed by measurement of fluorescence at an excitation wavelength of 485 nm and emission wavelength of 525 nm (slits 5 nm) in a Perkin-Elmer LS-5 spectrofluorimeter. Under these conditions, background fluorescence from fractions prepared from unlabelled platelets was negligible (comprising less than 1% of the total fluorescence).

Miscellaneous. NADH-dependent cytochrome c re-

ductase (antimycin A-insensitive) was measured as described [26]. Monoamine oxidase was measured as described by Wurtman & Axelrod [27]. Lactate dehydrogenase was assayed with a kit from Sigma Chemical Co. (340-UV); all fractions were diluted 5–50-fold in 0.1% Triton X-100 before this assay. Protein was measured as described by Lowry *et al.* [28], with bovine serum albumin as the standard. Sorbitol was included in the standard curve where interference was found to be significant.

Electron microscopy

Membrane fractions were pelleted by centrifugation at 130 000 g_{max} for 35 min at 4 °C, followed by resuspension in a few drops of warm 2% gelatin in phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.0). Thin sections were then prepared by the method described by Day *et al.* [29] and were examined under either a Philips or Zeiss transmission electron microscope.

Data analysis

Values are means $\pm$ S.E.M. unless otherwise indicated. Statistical differences between groups were determined by unpaired t tests, with a P value of 0.05 being considered a significant difference.

RESULTS

Distribution of α_2 -adrenergic receptor- G_i -adenylate cyclase system

Sorbitol-density-gradient centrifugation of homogenates of stored platelets yielded three major bands of turbidity on visual inspection. These three bands corresponded to three peaks of [3H]yohimbine binding (α_2 -adrenergic receptor), termed light (L), medium (M) and heavy (H) on the basis of their sedimentation behaviour (Fig. 1a). The relative amounts of [3H]yohimbine binding in L, M and H varied somewhat from experiment to experiment. Fig. 1(a) shows that the receptor and G_i sediment together on the gradients, but adenylate cyclase activity is virtually absent from the L fraction (see below). The enrichment of the α_2 -adrenergic receptor and G_i in the L fraction and their co-migration with the FITC-lectin (surface label) suggest that they are confined to the plasma membrane. There was no difference in the sedimentation pattern of protein, α_2 -adrenergic receptor or G_i with or without lectin pretreatment of the platelets (results not shown).

Fig. 1(b) shows the distribution of protein, monoamine oxidase and antimycin-A-insensitive NADH-dependent cytochrome c reductase for the same gradient shown in Fig. 1(a). Although not determined for this particular experiment, the cytosolic marker lactate dehydrogenase has the highest activity in fractions 1 or 2 in such gradients and is virtually absent in fractions 5 and above. The markers for mitochondria (monoamine oxidase) and for intracellular membranes (antimycin-A-insensitive NADH-dependent cytochrome c reductase) were most enriched in the peak of intermediate density (M).

The assignment of subcellular fractions was confirmed and the location of platelet α -granules and dense core granules determined by electron microscopy. Electron-microscopic examination of thin sections of pooled membranes from fractions L, M and H of stored platelets revealed a distinctive ultrastructural appearance in each fraction, similar to that reported for similar fractions by

Fig. 1. Subcellular distribution of platelet membrane markers

Washed platelets from 4 units of platelet concentrates were incubated with $20 \mu\text{g}$ of FITC-labelled lentil lectin/ml, washed again, frozen, homogenized and centrifuged as described in the Materials and methods section. Sorbitol-gradient (30–60%) centrifugation was performed, and membranes were collected from the resulting fractions by dilution and centrifugation for 30 min at $145000 g$. Membrane pellets were resuspended in buffer (50 mM-Tris/HCl/1 mM-EGTA, pH 7.6) and enzyme markers were measured as described in the Materials and methods section. (a) Activities of the α_2 -receptor/adenylate cyclase system markers (and their maximal activity) include: $[^3H]$ yohimbine binding ($\blacksquare$; 510 fmol/ml), pertussis-toxin substrate ($\circ$; 12 pmol/ml), FITC-labelled lectin ($\blacklozenge$; arbitrary fluorescence units) and basal adenylate cyclase ($\diamond$; 43 pmol/min per ml). (b) General membrane markers include: protein ($\blacktriangle$; 2.3 mg/ml), the mitochondrial marker monoamine oxidase ($\bullet$; 43 pmol/min per ml) and the endoplasmic-reticulum marker NADH-dependent cytochrome c reductase ($\triangle$; 290 nmol/min per ml).

Day *et al.* [29]. The L fraction showed a uniform collection of small (diameter $0.03\text{--}0.3 \mu\text{m}$), unilamellar, empty vesicles. The M fraction had the most heterogeneous appearance, containing: (1) large structures ($>2 \mu\text{m}$ in diameter) resembling intact or nearly intact platelets; (2) mitochondria, both intact and swollen; (3) unilamellar vesicular structures $0.2\text{--}1.0 \mu\text{m}$ in diameter, most of which were filled with an amorphous granular substance; (4) a wide variety of miscellaneous structures. The H fraction contained: (1) much amorphous granular material; (2) numerous small ($0.1\text{--}0.3 \mu\text{m}$ in diameter) single-membraned vesicles filled with a uniform electron-dense substance; (3) some recognizable mitochondria.

The heavy peak (H) showed substantial protein, but

was least enriched for all of the biochemical markers measured. It contained the bulk of the dense core and α -granules, by electron-microscopic evaluation. These results indicate that the L fraction is substantially enriched in plasma membranes, the H fraction contains dense core and α -granules as well as mitochondria and other membranes, and the M fraction is relatively enriched in intracellular membranes and mitochondria. Our separation of the plasma-membrane markers from the intracellular marker antimycin A-insensitive NADH-dependent cytochrome c reductase was not complete. Menashi *et al.* [30] and Fauvel *et al.* [31] also observed that it was difficult to separate intracellular membranes from plasma membranes with gradients run at neutral pH. The latter group [31] showed that at alkaline pH (9.6) there was a much improved separation of intracellular-membrane markers from the plasma membrane. Unfortunately, alkaline pH inactivates pertussis-toxin substrate activity [32], with effects observed at pH values as low as 10.

Determination of soluble G_i

Since the samples in gradient fractions in the preceding experiments were pelleted before assay for G_i , soluble pertussis-toxin substrates would not have been detected. Measurements of pertussis-toxin substrate activity in supernatants and total membrane fractions of platelets with and without prior adrenaline treatment are shown in Fig. 2(a). To be sure that soluble α subunits were not going undetected because of the requirement for the $\beta\gamma$ subunit for pertussis-toxin labelling, purified $\beta\gamma$ subunit from bovine brain was added to the samples. Although $[^{32}P]NAD^+$ incorporation into resolved α_o was markedly enhanced, there was no change in the labelling of either the membrane extracts or the supernatant samples from the platelets upon addition of $\beta\gamma$. To rule out the possibility of an inhibitor of the pertussis-toxin reaction in the supernatant samples, equal volumes of supernatant and membrane extract were mixed, and no inhibition of labelling of the membrane substrate was observed (results not shown).

Because of the very small amount of pertussis-toxin substrate in the supernatants, scintillation counting of supernatant samples even from the best experiment gave values (102 ± 3 c.p.m.) that were not reliably above background levels (93 ± 4 c.p.m.), whereas membrane samples gave easily measureable radioactivity (327 ± 14 c.p.m.). Values for adrenaline-treated platelets were not significantly different, with supernatant radioactivity of 104 ± 9 and a membrane value of 322 ± 6 c.p.m. In four experiments, with different background counts, the values were 17 ± 25 c.p.m. above background without and 21 ± 22 c.p.m. with adrenaline pretreatment. Although the reliability of these values for precise quantification of the amount of soluble pertussis-toxin substrate is limited, we can say from the scintillation-counting data that there is not more than approx. 3–5% of total pertussis-toxin substrate in the soluble phase of platelet homogenates. Densitometer scans of autoradiograms are shown in Fig. 2(b). By determination of the area under the peaks in such scans, there is $2.2 \pm 0.3\%$ as much pertussis-toxin substrate activity in the supernatant fractions as in the membranes. For adrenaline-pretreated platelets the value was not significantly different, $3.8 \pm 2.2\%$ ($P > 0.2$, unpaired *t* test).

The apparent depletion of adenylate cyclase in the

Fig. 2. Determination of pertussis-toxin substrates in platelet cytosol

(a) PRP was incubated for 3 h without or with 100 μ M-adrenaline (Epi) as described in the Materials and methods section. Platelets were isolated, frozen and homogenized, and the homogenates were centrifuged for 1 h at 100 000 g. The membrane pellet was resuspended in a volume of Tris/EGTA buffer equal to that of the supernatant, and cholate extracts of membrane (P) and supernatant (S) were prepared. The extracts were incubated with pertussis toxin and [32 P]NAD $^{+}$ in the absence and presence of 41 ng of purified $\beta\gamma$ subunit. Control lanes (Blank) included pertussis toxin, [32 P]NAD $^{+}$ and reaction cocktail, but no platelet extract. Also shown are 227 ng of purified α_o subunit with or without $\beta\gamma$. SDS/polyacrylamide-gel electrophoresis was performed, followed by autoradiography. (b) The autoradiogram from a similar experiment for which the background was cleaner was scanned for absorption (white light). The top traces are pertussis-toxin labelling of the membrane extracts from non-treated (–Epi) and adrenaline-treated (+Epi) platelets. The bottom two traces in each case are scans of duplicate lanes from the supernatant (Sups) fraction.

light membrane fraction (L) was unexpected, and was explored further. To test the possibility that this phenomenon was an artifact owing to the 24–36 h storage of platelets obtained from the Red Cross before processing, platelets prepared from freshly drawn blood were studied. Fig. 3 shows the gradient distribution of the α_2 -adrenergic receptor, G_i and adenylate cyclase from such an experiment. These platelets were not pretreated with FITC-lectin. Fractionation of platelets from freshly drawn blood consistently yielded only two peaks of [3 H]yohimbine binding and G_i , as opposed to the three peaks obtained with platelets from the Red Cross. When two peaks were obtained, the lighter of the two was termed 'L' and the heavier of the two 'MH',

Fig. 3. Subcellular distribution of α_2 receptor, G_i and adenylate cyclase in fresh platelets

Platelets from 400 ml of freshly drawn blood were isolated, washed, frozen and homogenized as described in the Materials and methods section. Then 2 ml of a low-speed-supernatant fraction was layered on a 30–60%–sorbitol gradient, centrifuged, and membrane fractions were collected as in Fig. 1. Activities of the α_2 -receptor/adenylate cyclase system markers (and their maximal activity) were: [3 H]yohimbine binding (■; 638 fmol/ml), pertussis-toxin substrate (○; 57.9 pmol/ml) and basal adenylate cyclase (◇; 72.9 pmol/min per ml); □, UK 14304 binding (175 fmol/ml).

because it migrated at a sorbitol density intermediate between those of the M and H fractions. The relative depletion of adenylate cyclase in the light fraction is nevertheless consistent with the results obtained with stored platelets.

The virtually identical distribution of [3 H]yohimbine binding and G_i in experiments with fresh platelets is confirmed quantitatively by calculating the ratio of the peak G_i concentration to that of the α_2 -adrenergic receptor. For three experiments the molar ratio of [32 P]NAD $^{+}$ incorporated to [3 H]yohimbine bound is 81 ± 16 for the L fraction and 70 ± 20 for MH. These values are similar to the ratio of 62 previously reported for plasma membranes purified from sonicated platelets [15]. The relative enrichment of [3 H]yohimbine binding and G_i in the L and MH fractions is also very similar, 2.6 and 2.9 respectively ($n = 2$). In contrast, the adenylate cyclase specific activity is lower in the L fraction, so that the relative specific activity in L compared with MH is 0.50, which quantitatively confirms the depletion seen in Figs. 1(a) and 3.

Latency of adenylate cyclase

One phenomenon that could explain the apparent lack of adenylate cyclase activity in the L fraction is the existence of permeability barriers in the form of sealed vesicles. The highly charged [32 P]ATP substrate would then be unable to enter the sealed vesicle to reach the catalytic site of adenylate cyclase on its inside surface. In an attempt to break down permeability barriers, membranes were preincubated with various concentrations of the non-ionic detergent Lubrol PX before an assay of forskolin-stimulated adenylate cyclase activity. Fig. 4 shows the result of such an experiment. There was a 3-fold increase in adenylate cyclase activity in the L fraction after treatment with 0.1 % Lubrol PX. This concentration

Fig. 4. Effect of Lubrol on adenylate cyclase activity in platelet membrane fractions

Pooled L (—), M (....) and H (---) fractions from a preparation similar to that in Fig. 1 were collected by centrifugation and resuspended in TME (50 mM-Tris/HCl/10 mM-MgCl₂/1 mM-EGTA, pH 7.6) buffer. Membranes were incubated with the indicated concentrations of Lubrol PX on ice for 1 h before measurement of adenylate cyclase activity in the presence of 10 μ M-forskolin. Data are expressed as percentage of the control values in the absence of Lubrol.

Fig. 5. Effect of adrenaline pretreatment on subcellular distribution of α_2 receptor and G_i

PRP from 400 ml of blood was prepared and treated with adrenaline as described in the Materials and methods section. Sucrose-gradient separation of the total homogenate of adrenaline-treated (■, ●) and control (□, ○) platelets was performed as described for Fig. 3. [³H]-Yohimbine binding (a) and pertussis-toxin substrate activity (b) were measured on membranes pelleted from each fraction.

of Lubrol significantly permeabilizes the platelet membranes, as >75 % of trapped [¹⁴C]sucrose is released by incubation for 1 h with 0.1 % Lubrol (M. A. Zamorski, unpublished work). In the M and H fractions there was respectively no effect and a small decrease in activity.

Effect of pretreatment of intact platelets with adrenaline

To identify changes in the subcellular distribution of the α_2 -adrenergic receptor or G_i after desensitization, we preincubated platelets prepared from freshly drawn blood with 100 μ M-adrenaline for 3 h at room temperature, followed by washing and fractionation as described above. The gradient distributions of the α_2 -adrenergic receptor and G_i for such an experiment are shown in Fig. 5. Adenylate cyclase activity was not measured. There is no difference in the α_2 -adrenergic receptor distribution between the control and adrenaline-treated platelets, nor is there a significant difference in the overall yield of this marker between the two gradients (1.81 versus 1.84 pmol). There is no significant difference in either the G_i distribution or in the yield of this marker between the two gradients (68 versus 73 pmol). Treatment of platelets with 100 μ M-adrenaline for 5 min also did not result in any change in the amount of membrane G_i activity or the appearance of G_i activity in supernatant fractions (results not shown).

DISCUSSION

In this study we report the subcellular distribution of the inhibitory guanine-nucleotide-binding protein G_i , the α_2 -adrenergic receptor and adenylate cyclase in human platelets. The localization of only 2–4 % of G_i in the soluble fraction is a significant finding. In other systems, more substantial amounts of G-proteins are released into a soluble form. The G_i homologue transducin from retinal rod outer segments is quantitatively released in a soluble form on treatment with light and guanine nucleotides [33]. In neutrophils [8] and liver membranes [9] guanine-nucleotide-binding activity is detected in a cytosol fraction. Isoprenaline treatment of S49 lymphoma cells results in the release of approx. 50 % of G_s into the cytosol [10]. In neutrophils, much smaller proportions (5–10 %) of the pertussis-toxin substrates and G_i -like immunoreactivity are found in soluble fractions [8,34], consistent with the findings reported here for platelets. The minimal release of G_i into cytosolic fractions compared with G_s and the low-molecular-mass G-proteins may be due to the fatty acylation of G_i , which is not seen for G_s [35]. Thus the notion that there is wholesale movement of G_i to the cytosol does not appear to be the case in human platelets or neutrophils.

Despite the similar or slightly smaller quantitative amounts of G_i in a soluble fraction, our results in platelets differ in certain respects from those of Rotrosen *et al.* [34] and Bokoch *et al.* [8] in neutrophils. We do not see any specific localization of G_i in the platelet granule fraction (H) over and above the amount resulting from contamination with plasma membranes. If 30 % of the pertussis-toxin substrate in the platelet were localized in granules, there would be a significant discrepancy between the distribution of pertussis-toxin substrate activity and the fluorescence lectin label. Activation of platelets with adrenaline does not alter the amount or distribution of G_i (Fig. 5), but activation with thrombin does decrease pertussis-toxin substrate activity ([36,37]; J. C. Ferraro,

unpublished work), as was seen in neutrophils with chemotactic peptide [34]. However, the data in neutrophils are conflicting on both of these points, too [8,34].

The pertussis-toxin substrate method used here for detecting G_i in platelet extracts has some limitations. First, it cannot distinguish among the multiple substrates that are present in mammalian cells [38–40]. Platelets do not contain G_o , the predominant G-protein in brain. G_{12} is the major pertussis-toxin substrate in platelets, with G_{13} being present in a much smaller amount [41,42]. Thus our data primarily reflect the distribution of α_{12} . Second, free α subunits will not be detected, because they are not efficient substrates for pertussis toxin [38]. This does not confound our conclusions, because addition of $\beta\gamma$ subunit does not increase the labelling by pertussis toxin in either membrane extracts or cytosol fractions (Fig. 2). Third, pertussis toxin does not label all of the G_i protein present. With soluble G_i and G_o , 50% or more of the protein can be modified by ADP-ribosylation [35]. The inefficient labelling of pertussis-toxin substrates is a much greater problem in platelet membranes and permeabilized platelets. Under the usual labelling conditions, less than 5% of the pertussis-toxin substrate in platelet membranes is ADP-ribosylated (M. H. Kim & R. R. Neubig, unpublished work). In permeabilized platelets approx. 400 ADP-ribose moieties are incorporated per platelet [43]. This is only 2–5% of the amount of pertussis-toxin substrate expected, given the 60-fold excess of pertussis-toxin substrate over α_2 receptors in extracts of platelet membranes ([15], and see above). With the method used here of solubilization in sodium cholate and Lubrol before ADP-ribosylation, we should be detecting the majority of the pertussis-toxin substrate in the platelet.

A preliminary report on the G_i protein in platelets shows that adrenaline, but not thrombin, results in a shift of immunoreactive α_i from a Triton-soluble to a Triton-insoluble fraction [42]. Our results show that there is no bulk shift in G_i from one subcellular compartment to another after treatment with adrenaline, so the distinction between Triton-soluble and Triton-insoluble forms is likely to represent a local change in the interactions of the G_i with cytoskeletal elements, rather than gross shifts in the localization of the protein in the cell. The lack of effect of adrenaline on the distribution of G_i could be due to several reasons. Acute desensitization of platelet responses by adrenaline is not accompanied by a decrease in receptor number, or even a decrease in adenylate cyclase inhibition [44]. We also have seen no decrease in the recovery of [3 H]yohimbine-binding sites after adrenaline pretreatment (see the Results section). Alternatively, a loss of G_i could be occurring with desensitization, but, if the amount lost is roughly stoichiometric with the amount of α_2 receptor, then the large excess of G_i over α_2 receptors makes the amount of G_i lost difficult to detect.

Adenylate cyclase has generally been considered to be a plasma-membrane enzyme. By the free-flow-electrophoresis technique, this enzyme was found to predominate in the plasma-membrane fraction [30], but the specific activities reported by that group were at least 50-fold lower than those typically seen in our laboratory, so this result is hard to reconcile with our findings. Using a histochemical technique, one group reported the localization of prostaglandin E_1 -stimulatable adenylate cyclase to the dense tubular system in human platelets

[45,46]. Our data suggest the existence of a pool of adenylate cyclase activity in a non-plasma-membrane fraction; however, the complex effects of Lubrol on the enzyme activity prevent us from definitively ruling out latency as the sole mechanism. More direct measures of the distribution of adenylate cyclase protein rather than activity will be necessary to clarify this issue. The degree of latency of adenylate cyclase in the light-membrane/vesicle preparation suggests that at least two-thirds of this fraction consists of sealed right-side-out vesicles. This property may prove useful in studies of the sidedness of various plasma membrane components.

In astrocytoma cells [22] and frog erythrocytes [23], the distribution of β -adrenergic receptors changes after prolonged treatment of the cells with agonist. Stadel *et al.* [23] reported a decrease in the number of surface receptors and the recovery of most of these 'lost' receptors in the form of intracellular vesicles containing neither G_s or adenylate cyclase activity. Agonist binding and response data indicate that these receptors are 'uncoupled' from G_s . In contrast, desensitization of the α_2 -agonist-mediated platelet-aggregation response [44] occurs distal to the regulation of adenylate cyclase. Our findings also indicate that uncoupling of the α_2 receptors and G_i does not occur after agonist exposure in human platelets.

To explain the presence of low-affinity α_2 -agonist binding in the presence of excess G_i , we have proposed that either the receptor or G_i is heterogeneous, or that these two elements are 'compartmentalized' in the cell in such a way that not all of the G_i pool is capable of interacting with the receptor [15,47]. The studies reported here were designed to test the compartmentation hypothesis. The co-migration of the α_2 -adrenergic receptor and G_i in density-gradient centrifugation points away from compartmentation in distinct subcellular fractions. If compartmentation is the mechanism, it would have to be at the level of lateral domains of the membrane, as recently reported for human neutrophils [48] or tight interactions of the G_i with other receptors or perhaps cytoskeletal elements ([49], and see above). The hypotheses of receptor heterogeneity and cytoskeletal interactions require investigation.

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REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
2. Limbird, L. E. (1988) *FASEB J.* **2**, 2686–2695
3. Exton, J. H. (1988) *FASEB J.* **2**, 2670–2676
4. Fain, J. N., Wallace, M. A. & Wojcikiewicz, R. J. H. (1988) *FASEB J.* **2**, 2569–2574
5. Garcia-Sainz, J. A. (1988) *Trends Pharmacol. Sci.* **9**, 271–272
6. Rodbell, M. (1985) *Trends Biochem. Sci.* **10**, 461–464
7. Chabre, M. (1985) *Annu. Rev. Biophys. Biophys. Chem.* **14**, 331–360

8. Bokoch, G. M., Bickford, K. & Bohl, B. P. (1988) *J. Cell Biol.* **106**, 1927–1936
9. Lynch, C. J., Morbach, L., Blackmore, P. F. & Exton, J. H. (1986) *FEBS Lett.* **200**, 333–336
10. Ransnäs, L. A., Svoboda, P. & Insel, P. A. (1988) *Pharmacologist* **30**, A101 (abstr.)
11. Deckmyn, H., Tu, S. M. & Majerus, P. W. (1986) *J. Biol. Chem.* **261**, 16553–16558
12. Molina y Vedia, L. & Lapetina, E. G. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 868–870
13. Steer, M. L. & Wood, A. (1979) *J. Biol. Chem.* **254**, 10791–10797
14. Jakobs, K. H., Saur, W. & Schultz, G. (1978) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **302**, 285–291
15. Neubig, R. R., Gantzios, R. D. & Brasier, R. S. (1985) *Mol. Pharmacol.* **28**, 475–486
16. Hoffman, B. B., Michel, T., Kilpatrick, D. M., Lefkowitz, R. J., Tolbert, M. E., Gilman, H. & Fain, J. N. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4569–4573
17. Hoffman, B. B., Michel, T., Brenneman, T. B. & Lefkowitz, R. J. (1982) *Endocrinology (Baltimore)* **110**, 926–932
18. Kurose, H., Katada, T., Amano, T. & Ui, M. (1983) *J. Biol. Chem.* **258**, 4870–4875
19. Kim, M. H. & Neubig, R. R. (1987) *Biochemistry* **26**, 3664–3672
20. Thomsen, W. J., Jacquez, J. A. & Neubig, R. R. (1988) *Mol. Pharmacol.* **34**, 814–822
21. Convents, A., DeBacker, J.-P., Convents, D. & Vauquelin, G. (1987) *Mol. Pharmacol.* **32**, 65–72
22. Harden, T. K., Cotton, C. U., Waldo, G. L., Lutton, J. K. & Perkins, J. P. (1980) *Science* **210**, 441–443
23. Stadel, J. M., Strulovici, B., Nambi, P., Lavin, T. N., Briggs, M. M., Caron, M. G. & Lefkowitz, R. J. (1983) *J. Biol. Chem.* **258**, 3032–3038
24. Neubig, R. R. & Szamraj, O. (1986) *Biochim. Biophys. Acta* **854**, 67–76
25. Bokoch, G. M., Katada, T., Northup, J. K., Ui, M. & Gilman, A. G. (1984) *J. Biol. Chem.* **259**, 3560–3567
26. Tolbert, N. E. (1974) *Methods Enzymol.* **31**, 734–746
27. Wurtman, R. & Axelrod, J. (1963) *Biochem. Pharmacol.* **12**, 1439–1441
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
29. Day, H. J., Holmsen, H. & Hovig, T. (1969) *Scand. J. Haematol. (Suppl.)* **7**, 3–35
30. Menashi, S., Weintraub, H. & Crawford, N. (1981) *J. Biol. Chem.* **256**, 4095–4101
31. Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S. & Douste-Blazy, L. (1986) *Biochim. Biophys. Acta* **856**, 155–164
32. Kim, M. H. & Neubig, R. R. (1985) *FEBS Lett.* **192**, 321–325
33. Baehr, W., Morita, E. A., Swanson, R. J. & Applebury, M. L. (1982) *J. Biol. Chem.* **257**, 6452–6460
34. Rotrosen, D., Gallin, J. I., Spiegel, A. M. & Malech, H. L. (1988) *J. Biol. Chem.* **263**, 10958–10964
35. Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G. & Sefton, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7493–7497
36. Lapetina, E. G., Reep, B. & Chang, K. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5880–5883
37. Halenda, S. P., Volpi, M., Zavoico, G. B., Shaafi, R. I. & Feinstein, M. B. (1986) *FEBS Lett.* **204**, 341–346
38. Neer, E. J., Lok, J. M. & Wolf, L. G. (1984) *J. Biol. Chem.* **259**, 14222–14229
39. Mumby, S., Pang, I. H., Gilman, A. G. & Sternweis, P. C. (1988) *J. Biol. Chem.* **263**, 2020–2026
40. Beals, C. R., Wilson, C. B. & Perlmutter, R. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7886–7890
41. Nagata, K., Katada, T., Tohkin, M., Itoh, H., Kaziro, Y., Ui, M. & Nozawa, Y. (1988) *FEBS Lett.* **237**, 113–117
42. Crouch, M. F., Winegar, D. A. & Lapetina, E. G. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1776–1780
43. Banga, H. S., Walker, R. K., Winberry, L. K. & Rittenhouse, S. E. (1988) *Biochem. J.* **252**, 297–300
44. Motulsky, H. J., Shattil, S. J., Ferry, N., Rozansky, D. & Insel, P. A. (1986) *Mol. Pharmacol.* **29**, 1–6
45. Cutler, L. S., Feinstein, M. B., Rodan, G. A. & Christian, C. P. (1981) *Histochem. J.* **13**, 547–554
46. Cutler, L. S., Christian, C. P. & Feinstein, M. B. (1985) *Biochim. Biophys. Acta* **845**, 403–410
47. Neubig, R. R., Gantzios, R. D. & Thomsen, W. J. (1988) *Biochemistry* **27**, 2374–2384
48. Jesaitis, A. J., Bokoch, G. M., Tolley, J. O. & Allen, R. A. (1988) *J. Cell Biol.* **107**, 921–928
49. Carlson, K. E., Woolkalis, M. J., Newhouse, M. G. & Manning, D. R. (1986) *Mol. Pharmacol.* **30**, 463–468

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