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Characterization of a G Protein-Coupled Receptor for Nicotinic Acid

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ABSTRACT

Nicotinic acid is a lipid-lowering agent widely used to treat hypertriglyceridemia and to elevate low high density lipoprotein levels. However, the underlying mechanisms are poorly understood. In this study, G protein activation by nicotinic acid and derivatives was assessed as stimulation of guanosine 5′-(γ-[^{35}S]-thio)triphosphate ([^{35}S]GTPγS) binding, and [^{3}H]nicotinic acid was used for specific labeling of binding sites. Nicotinic acid (EC_{50} ~1 μM) stimulated [^{35}S]GTPγS binding in membranes from rat adipocytes and spleen, but not from other tissues. G protein activation in adipocyte membranes in the presence of maximally activating concentrations of the selective A_1 adenosine receptor agonist 2-chloro-N^6-cyclopentyladenosine and nicotinic acid was almost additive, indicating that G proteins of mostly distinct pools were activated by these agonists. G protein activation by nicotinic acid and related substances in spleen and adipocytes revealed identical pharmacological profiles. [^{3}H]Nicotinic acid specifically detected guanine nucleotide-sensitive binding sites of identical pharmacology in adipocyte and spleen membranes. The site of action of nicotinic acid is distinct from other G protein-coupled receptors. These data indicate that nicotinic acid most probably acts on a specific G protein-coupled receptor.

Hypercholesterolemia is a relevant risk factor with regard to the development of atherosclerotic diseases. Inadequate response to dietary therapy may require the administration of lipid-lowering drugs. One strategy to decrease elevated levels of lipids in blood is the inhibition of lipolysis in adipose tissue. This approach involves regulation of hormone-sensitive lipase, which is the rate-limiting enzyme in lipolysis. Lipolytic agents, e.g., β-adrenergic agonists, increase cellular levels of cyclic AMP, which, in turn, activates protein kinase A and leads to phosphorylation and activation of hormone-sensitive lipase (Yeaman, 1990). In contrast, various antilipolytic agents, e.g., adenosine, act by lowering intracellular cyclic AMP levels (Schwabe et al., 1973).

Nicotinic acid and the metabolically stable derivative 5-methylpyrazine-2-carboxylic acid-4-oxide (acipimox; Fig. 1) are commonly used drugs for the treatment of hyperlipidemia (DiPalma and Thayer, 1991). The benefits of nicotinic acid in the treatment or prevention of atherosclerotic cardiovascular disease have been documented in six major clinical trials (for review, see Guyton, 1998). Among other hypolipidemic agents, nicotinic acid seems unique because of its potential to increase high density lipoprotein cholesterol to a greater extent than other drugs (Kwiterovich, 1998). Nicotinic acid and related heterocyclic compounds inhibit lipolysis in adipose tissue. This action of nicotinic acid and analogs involves a decrease in cellular cyclic AMP levels by inhibition of adipocyte adenylyl cyclase (Aktories et al., 1980a) and stimulation of a high-affinity GTPase in fat cell membranes (Aktories et al., 1980b, 1982). The inhibition of adenylyl cyclase requires a functional G protein of the G_i/G_o family because GTPase activation as well as adenylyl cyclase inhibition are prevented by pertussis toxin (Aktories et al., 1983). In contrast, augmentation of insulin-stimulated glucose transport by nicotinic acid in rat adipocytes requires a pertussis toxin-sensitive G protein, but is probably independent from cyclic AMP (Kuroda et al., 1987; Honnor et al., 1992).

The most frequently observed side effect of nicotinic acid is skin flushing, which may be ameliorated by low doses of cyclooxygenase inhibitors. Cyclooxygenase inhibitors do not prevent the lipid-lowering actions of nicotinic acid (Kaijser et al., 1979), which indicates that these effects are mediated via distinct mechanisms, and cyclooxygenase products are not involved in the inhibition of lipolysis by nicotinic acid.

Although to date it is known that the hypolipidemic effects of nicotinic acid involve adenylyl cyclase inhibition via a pertussis toxin-sensitive G protein, knowledge about the pri-

ABBREVIATIONS: acipimox, 5-methylpyrazine-2-carboxylic acid-4-oxide; CCPA, 2-chloro-N^6-cyclopentyladenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; GDPβS, guanosine 5-(β-thio)diphosphate; GTPγS, guanosine 5′-(γ-thio)triphosphate; UK 14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine; *R*(+)-WIN 55,212-2 mesylate, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; *R*-PIA, [^{3}H]*R*-N^6-phenylisopropyladenosine.

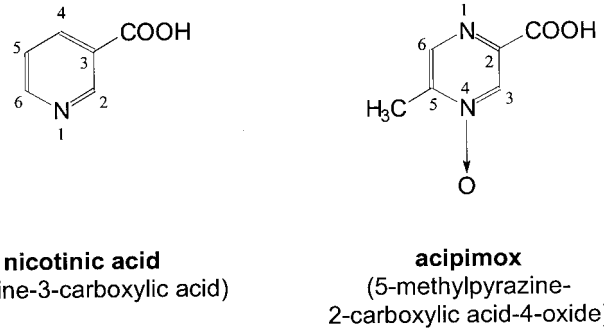
mary site and mechanism of action of nicotinic acid is scarce. In the present study, we have characterized the requirements for G protein activation by nicotinic acid in membranes of rat adipocytes. Structure-activity relationships for G protein activation by nicotinic acid and analogs were investigated. In addition, the site of action of nicotinic acid and related heterocycles was characterized with respect to localization in rat tissues. For the first time, a distinct membrane-associated nicotinic acid binding site of appropriate pharmacology has been identified in direct binding studies. Therefore, it must be concluded that the primary site of action of nicotinic acid is a specific membrane-bound receptor.

Experimental Procedures

Materials. Guanosine 5′-(γ-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS; 1250 Ci/mmol) was obtained from NEN Life Science Products (Cologne, Germany). [5,6-^{3}H]Nicotinic acid (50–60 Ci/mmol) was purchased from Biotrend (Cologne, Germany). 2-Chloro-N^6-cyclopentyladenosine (CCPA) and pyridine-2,3-dicarboxylic acid (quinolinic acid) came from RBI/Sigma (Deisenhofen, Germany). Imidazole-4-acetate was purchased from Tocris (Bristol, England). 6-Methylnicotinic acid, furane-3-carboxylic acid, 2-hydroxynicotinic acid, imidazole-4-carboxylic acid, indole-3-carboxylic acid, 2-methylnicotinic acid, 5-methylpyrazine-2-carboxylic acid, nicotinic acid-1-oxide, piperidine-3-carboxylic acid, pyrazine-2,3-dicarboxylic acid, pyrazine-2-carboxylic acid, 3-pyridine-acetic acid, 3-(3-pyridine)-propionic acid, and quinoline-3-carboxylic acid came from Sigma-Aldrich (Deisenhofen, Germany). Benzoic acid, collagenase (from *Clostridium histolyticum*, type II), isonicotinic acid (pyridine-4-carboxylic acid), nicotinic acid (pyridine-3-carboxylic acid), nicotinamide, pertussis toxin, NAD, GTPγS, bovine serum albumin (fraction V), piperazine-2-carboxylic acid, pyridine-3,5-dicarboxylic acid, and quinoline-3-carboxylic acid (quinaldic acid) were purchased from Sigma (Deisenhofen, Germany). Adenosine deaminase, ATP, GDP, guanosine 5-(β-thio) diphosphate (GDPβS), dithiothreitol, and 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS) were purchased from Roche Biochemicals (Mannheim, Germany). Acipimox was a generous gift from Pharmacia-Upjohn (Erlangen, Germany). All other materials were from standard sources and of the highest purity commercially available.

Synthesis. 4-Methylnicotinic acid and 5-methylnicotinic acid were synthesized according to a published procedure (Clarke et al., 1984). Pyridazine-4-carboxylic acid was prepared as described (Leanza et al., 1953).

Membrane Preparations. Male Wistar rats (6–8 weeks old, body weight ~150 g) were anesthetized with ether and decapitated. Isolated fat cells from epididymal, omental, and renal fat pads were prepared by collagenase digestion according to the method of Rodbell (1964) in 123 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.5 mM $MgSO_4$, 32 mM $NaHCO_3$, 11 mM glucose, and 1% bovine serum albumin, pH 7.4, at 37°C. Membrane preparations from isolated adipocytes were performed according to McKeel and Jarett (1970). The membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.4, frozen in liquid nitrogen and stored at −75°C. Membranes from other rat organs (forebrain, spleen, liver, kidney, testis, heart, lung) were prepared after homogenization of tissues in a 9-fold volume of ice-cold 0.32 M sucrose with a polytron (Kinematika, Luzern, Switzerland) for 20 s (setting 6). All subsequent steps were performed at 0 to 4°C. Homogenates were centrifuged for 10 min at 1000*g* [3200 rpm in a Beckman (Palo Alto, CA) JA-17 rotor]. The supernatants were collected and centrifuged for 40 min at 100,000*g* (37,000 rpm in a Beckman Ti-60 rotor). The pellet was resuspended in H_2O with a polytron, centrifuged for 30 min at 100,000*g,* and washed twice as described above with 50 mM Tris-HCl, pH 7.4. The membranes were resuspended in the same buffer, frozen in liquid nitrogen, and stored at −75°C. Protein concentrations were measured according to the method described by Peterson (1977), using bovine serum albumin as standard.



nicotinic acid
(pyridine-3-carboxylic acid)

acipimox
(5-methylpyrazine-2-carboxylic acid-4-oxide)

Fig. 1. Structures of the hypolipidemic agents nicotinic acid and acipimox.

[^{35}S]GTPγS Binding. G protein activation by agonists in rat membrane preparations was assessed as stimulation of [^{35}S]GTPγS binding as described previously (Lorenzen et al., 1993, 1996). Briefly, samples were incubated in a total volume of 100 μl containing 50,000 cpm (0.2 nM) [^{35}S]GTPγS, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 10 μM GDP, 0.5 U/ml adenosine deaminase, and 0.5% bovine serum albumin, if not indicated otherwise. Incubations were performed with 0.5 to 2 μg of membrane protein for 90 min at 25°C and were terminated by filtration over GF/B glass fiber filters (Whatman, Maidstone, England) followed by two 4-ml washes with ice-cold buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.02% CHAPS). Adenosine deaminase was added to all samples to remove endogenous adenosine, which leads to background activation of G proteins in the absence of exogenous agonists (Laitinen and Jokinen, 1998).

[^{3}H]Nicotinic Acid Binding. Equilibrium binding of [^{3}H]nicotinic acid to membranes from rat tissues was done with 50 to 100 μg of membrane protein per tube in a total volume of 250 μl in 50 mM Tris-HCl, pH 7.4, containing 0.02% CHAPS. In the presence of 0.02% CHAPS, nonspecific binding was lower than in its absence, with no effect on specific binding. In some experiments, $MgCl_2$ was added to a final concentration of 1 mM. Binding experiments were conducted, if not indicated otherwise, in the presence of 20 nM radioligand for 3.5 h at 25°C, according to previous time course experiments. Nonspecific binding was assessed in the presence of 100 μM acipimox. In kinetic experiments, dissociation of the radioligand was induced by addition of acipimox (final concentration 100 μM). Separation of membrane-bound from unbound radioligand was done by filtration of the samples through nitrocellulose filters and two washing steps, each with 4 ml of 50 mM Tris-HCl (pH 7.4) with 0.02% CHAPS.

ADP-Ribosylation of Membranes with Pertussis Toxin. Pertussis toxin (200 μg/ml) was preactivated in the presence of 50 mM dithiothreitol for 1 h at 20°C. ADP-ribosylation was performed for 1 h at 25°C with 20 μg of activated toxin/ml, 1 mg of protein/ml of adipocyte, or spleen membranes in 50 mM Tris-HCl pH 7.4, 3 mM NAD, 1 mM ATP, 2 mM GDP, and 25 mM dithiothreitol. Control membranes were subjected to identical treatment, but pertussis toxin was omitted. Adipocyte membranes were diluted 200-fold and used directly for [^{35}S]GTPγS binding experiments. The resulting concentrations of NAD (15 μM) and ATP (5 μM) did not interfere with the assay. Spleen membranes were washed three times with 50 mM Tris-HCl (pH 7.4) and finally resuspended in the same buffer for [^{3}H]nicotinic acid binding.

Data Analysis. Affinity (K_d) and maximum binding capacity (B_{max}) of nicotinic acid-induced stimulation of [^{35}S]GTPγS binding was calculated from nonlinear curve fitting with SigmaPlot (Jandel Scientific, Erkrath, Germany). EC_{50} values for stimulation of [^{35}S]GTPγS binding were calculated from fitting experimental results to sigmoid dose-response curves with SigmaPlot. K_{-1}, K_{obs}, and K_{+1} values from kinetic experiments were calculated by fitting bind-

ing data to monophasic association and dissociation curves with SigmaPlot. Data were fitted to a one-site model because fitting to two sites did not improve the fit. K_d, K_i, and B_{max} values from [^{3}H]nicotinic acid binding experiments were calculated through nonlinear curve fitting with the program SCTFIT (De Lean et al., 1982). Results were fitted to a one-site model if curve fitting to two sites did not improve the fit significantly ($p < 0.05$, *f*-test). EC_{50}, K_d, and K_i values are given as geometric means with 95% confidence limits from 3 to 10 experiments. All other data are arithmetic means ± S.E. Statistical analysis of differences was performed using the Student's *t* test. Multiple comparisons were performed after analysis of variance followed by the multiple comparison Student-Newman-Keuls test. Results were considered significantly different when $p < 0.05$.

Results

Nicotinic acid stimulates [^{35}S]GTPγS binding in adipocyte membranes, which reflects the GDP-GTP exchange reaction in G protein activation. The levels of maximum stimulation by nicotinic acid were 2- to 3-fold above basal levels in rat adipocyte membranes from epididymal, renal, and abdominal fat (Fig. 2A). Sensitivity to nicotinic acid did not differ in adipocyte membranes obtained from the different localizations. EC_{50} values of nicotinic acid in G protein activation were 1.05 (0.95–1.16) μM for adipocyte membranes from epididymal fat pads, 1.32 (0.63–2.74) μM in membranes from abdominal, and 1.61 (0.84–3.05) μM in membranes from renal fat pads. For further experiments, membranes from epididymal fat cells were used.

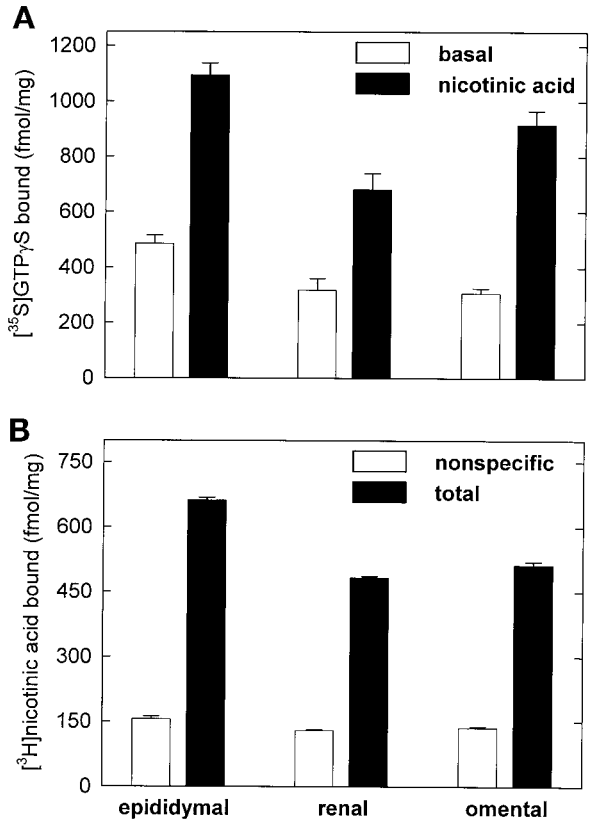


Fig. 2. Interaction of nicotinic acid with rat adipocyte membranes. [^{35}S]GTPγS binding (A) under basal conditions (open columns) and stimulated by 100 μM nicotinic acid (filled columns) was determined in adipocyte membranes (2 μg/tube) from fat cells of different localizations. Incubations were performed for 90 min at 25°C as described under *Experimental Procedures*. B, [^{3}H]nicotinic acid (20 nM) binding to membranes from adipocytes from fat pads of different localizations was measured after 3.5 h of incubation at 25°C. Total (filled columns) and nonspecific radioligand binding (in the presence of 100 μM acipimox; open columns) were determined using 75 μg of membrane protein. Results are means ± S.E.M. from three experiments.

The influence of nicotinic acid on the G protein activational state was further characterized in comparison to the effects of CCPA, which acts as a selective agonist of the G protein-coupled A_1 adenosine receptor in adipocytes. Stimulation of [^{35}S]GTPγS binding by nicotinic acid and CCPA showed an absolute requirement for the presence of GDP (Fig. 3). GDP decreases basal binding of the radioligand to a greater extent than binding in the presence of CCPA or nicotinic acid. The maximum of agonist-induced increases in [^{35}S]GTPγS binding were observed at 1 to 10 μM GDP (Fig. 3). Therefore, further experiments were performed in the presence of 10 μM this nucleotide. In the following experiments, we investigated whether nicotinic acid, via an unknown mechanism, and CCPA, via activation of A_1 adenosine receptors, stimulate [^{35}S]GTPγS binding to identical or distinct G protein pools. Concentration-dependent G protein activation by CCPA and nicotinic acid was measured in the absence or presence of a maximally stimulating concentration of the second agonist, and concentration-response curves are shown in Fig. 4. Nicotinic acid stimulated [^{35}S]GTPγS binding to adipocyte membranes to higher maximum levels than CCPA, albeit with a lower potency. In the presence of 10 μM CCPA, nicotinic acid led to a further increase in [^{35}S]GTPγS binding. Similarly, CCPA stimulated G protein activation also in the presence of 1 mM nicotinic acid. CCPA stimulated [^{35}S]GTPγS binding by 1086 ± 241 cpm/μg above basal levels; nicotinic acid stimulation was 1693 ± 170 cpm/μg of protein above nonstimulated binding (three experiments). The total stimulation by both agonists simultaneously present was 2216 ± 361 cpm/μg, which corresponds to 79.7% of the expected stimulation (2780 cpm/μg) if both agonists were fully additive. Therefore, we assume that nicotinic acid and CCPA activate G protein pools that are, to the greatest part, not identical. In agreement with this result, we found that the potencies of CCPA and nicotinic acid were identical in the absence or presence of the second agonist, indicating an absence of mutual regulation of both G protein activation pathways.

Structure-activity relationships were investigated for heterocyclic compounds related to nicotinic acid by assessing their potency and maximum stimulatory effects in G protein activation in adipocyte membranes (Table 1). Nicotinic acid was the most potent agent. The carboxyl group of nicotinic acid is essential for its stimulatory activity, because nicotinamide was virtually inactive in concentrations up to 1 mM. 3-Pyridine-acetic acid (EC_{50} 16.4 μM) and 3-(3-pyridine)-propionic acid (inactive up to 1 mM) were much less potent than nicotinic acid. Substitution of nicotinic acid with methyl groups in positions 5 or 6 greatly diminished potency (EC_{50} 5-methylnicotinic acid, 30.2 μM; 6-methylnicotinic acid, 72.6 μM). 2-Methyl- and 4-methyl-nicotinic acid were inactive. Other heterocyclic agents that also proved to stimulate G protein activation were pyridazine > pyrazine > furane derivatives. Although oxidation of the pyridine ring nitrogen and a 5-methyl-substituent diminish the potency of nicotinic acid approximately 60- and 20-fold, and pyrazine-2-carboxylic acid is also approximately 20-fold less potent than nico-

tinic acid, acipimox displayed only 7-fold lower potency than nicotinic acid in G protein activation (EC_{50} 10.3 μM). Isonicotinic acid, benzoic acid, indole-3-carboxylic acid, imidazole derivatives (imidazole-4-carboxylic acid, imidazole-4-acetate), quinoline derivatives (quinoline-2-carboxylic acid, quinoline-3-carboxylic acid), and compounds with two carboxyl groups (quinolinic acid, pyridine-3,5-dicarboxylic acid, pyrazine-2,3-dicarboxylic acid, pyridazine-4,5-dicarboxylic acid) did not enhance G protein activation. The nonaromatic analogs piperidine-3-carboxylic acid and piperazine-3-carboxylic acid were also inactive in concentrations up to 1 mM, indicating that an aromatic ring is required for the stimulatory effect.

Because nicotinic acid affects not only adipose tissues, we investigated whether activation of G proteins by this agent was detectable in membranes from other tissues. In addition to its effect in adipocyte membranes, nicotinic acid induced stimulation of [^{35}S]GTPγS binding only in spleen membranes, but not in membranes from forebrain, liver, kidney, testis, heart, or lung (Fig. 5). Further studies sought to determine whether the stimulatory site in adipocyte and spleen membranes were identical. With the exception of 5-methylpyrazine-2-carboxylic acid, which was ~3-fold more potent in G protein activation in spleen membranes than in adipocyte membranes, stimulation of [^{35}S]GTPγS binding in spleen and fat cell membranes displayed identical pharmacological profiles (Table 1). Therefore, we assume that these two sites are identical.

The site of action of nicotinic acid was further characterized by direct binding studies using [^{3}H]nicotinic acid as a radioligand. Nonspecific binding was defined as the binding in the presence of 100 μM acipimox. Specific binding of [^{3}H]nicotinic acid was found in adipocyte membranes from epididymal, renal, and abdominal fat (Fig. 2B). In agreement with the ability of nicotinic acid to activate G proteins only in membranes from fat cells and spleen, [^{3}H]nicotinic acid labeled sites in spleen membranes, but not in membranes prepared from other organs (Fig. 6). Kinetic experiments demonstrated that [^{3}H]nicotinic acid binding to spleen membranes was reversible and monophasic. Dissociation was induced by addition of 100 μM acipimox and yielded a kinetic K_d value of 12.3 nM (K_{-1} 4.392 $\pm$ 0.074 $\times$ 10^{-3} min^{-1}, dissociation $t_{1/2}$ 158 min, K_{obs} 11.61 $\pm$ 1.13 $\times$ 10^{-3} min^{-1}, K_{+1} 0.361 $\pm$ 0.0582 $\times$ 10^{-3} liter $\times$ $nmol^{-1}$ min^{-1}; mean values $\pm$ S.E.M. from three experiments).

Binding of [^{3}H]nicotinic acid to epididymal adipocyte and spleen membranes was saturable in a single component (Fig. 7). Nonspecific binding of the radioligand, as determined in the presence of 100 μM acipimox, amounted to approximately 30% of total binding at K_d. Affinities of [^{3}H]nicotinic acid were slightly lower in adipocyte membranes [K_d 43.5 (39.5–48.0) nM] than in spleen membranes [K_d 22.8 (18.6–27.9) nM], in agreement with the slightly higher potency of nicotinic acid in spleen membranes in G protein activation (Table 1). The densities of the [^{3}H]nicotinic acid binding site were somewhat higher in adipocyte membranes (B_{max} 1518 $\pm$ 115 fmol/mg) compared with spleen membranes (B_{max} 1078 $\pm$ 145 fmol/mg). The maximum possible G protein activation by CCPA and nicotinic acid was compared in [^{35}S]GTPγS saturation experiments performed in the absence or presence of these agonists. Saturation was performed as isotopic dilution of [^{35}S]GTPγS with unlabeled GTPγS (Fig. 8). The agonist-induced increases in GTPγS binding were fitted to saturation isotherms (Fig. 8, inset). The affinity of GTPγS in agonist-stimulated [^{35}S]GTPγS binding was 0.92 (0.76–1.10) nM for CCPA and 0.64 (0.44–0.94) nM for nicotinic acid. The maximum increase in GTPγS binding was somewhat higher for nicotinic acid (B_{max} 1755 $\pm$ 157 fmol/mg) compared with CCPA (1482 $\pm$ 104 fmol/mg).

The Scatchard plots of the [^{3}H]nicotinic acid saturation experiments, especially in spleen membranes (Fig. 7, upper panel), might indicate two sites, although curve fitting to two sites did not improve the fit significantly. We have therefore investigated in more detail whether [^{3}H]nicotinic acid labels several binding sites in spleen membranes. To enhance agonist binding, additional saturation experiments were performed in the presence of 1 mM $MgCl_2$. The affinity of [^{3}H]nicotinic acid was not different in the presence of $MgCl_2$ [K_d 25.5 (20.1–32.2) nM]. However, the maximum binding capacity was significantly higher in the presence than in the absence of Mg^{2+} ions (B_{max} 1555 $\pm$ 121 fmol/mg). [^{3}H]Nicotinic acid binding was guanine nucleotide-sensitive (Fig. 9). GTPγS was more effective than GDPβS in inhibition of radioligand binding. However, the effects of these guanine nucleotides were rather weak. The omission of CHAPS from the incubations did not increase the effects of GDPβS and GTPγS (not shown). The low efficacy of GTPγS and GDPβS is not attributable to the lack of Mg^{2+} ions, since identical results were obtained in the absence (Fig. 9) and presence of 1 mM $MgCl_2$ (not shown). The possibility that the low efficacy of the

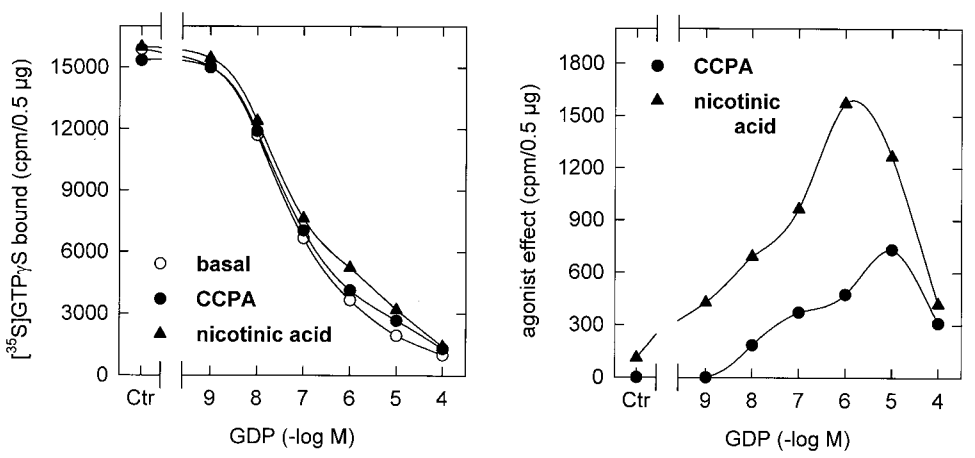


Fig. 3. Influence of GDP on basal and agonist-stimulated [^{35}S]GTPγS binding. Left, [^{35}S]GTPγS binding to epididymal adipocyte membranes (0.5 μg/tube) was determined in the presence of increasing GDP concentrations in the absence (○) or presence of 10 μM CCPA (●) or 1 mM nicotinic acid (▲) after 90 min of incubation at 25°C. Right, stimulation of [^{35}S]GTPγS binding by CCPA (●) and nicotinic acid (▲) above basal levels. Results from one of three experiments are shown.

guanine nucleotides was attributable to labeling of high- and low-affinity binding sites by [^{3}H]nicotinic acid was addressed in competition experiments with unlabeled nicotinic acid in the absence or presence of 10 and 100 μM GTPγS. All inhibition curves were monophasic. GTPγS led to a stepwise decrease in the affinity from 35.8 (27.6–46.4) nM under control conditions to 53.3 (45.6–62.2) nM in the presence of 10 μM and to 64.4 (50.7–81.7) nM in the presence of 100 μM this nucleotide (each $p < 0.05$ versus control). B_{max} values were significantly reduced to 53.9 ± 4.3% in the presence of 10 μM and to 34.1 ± 2.2% in the presence of 100 μM GTPγS. Almost identical results were obtained in the presence of 1 mM $MgCl_2$ (not shown).

Since the yield of membranes from rat spleen (~10 mg/animal) is considerably higher than that from epididymal adipocytes (~0.5 mg/animal), the binding site labeled with [^{3}H]nicotinic acid was characterized in more detail in rat spleen membranes. The pharmacological characteristics of the site labeled by [^{3}H]nicotinic acid were addressed in competition experiments with the heterocyclic compounds that had been used previously in G protein activation studies. All inhibition curves were strictly monophasic, indicating that only a single site was specifically labeled by [^{3}H]nicotinic acid. The rank orders of potency in inhibition of [^{3}H]nicotinic acid binding to spleen membranes and in stimulation of [^{35}S]GTPγS binding to adipocyte and spleen membranes were identical (Table 2). This indicates that [^{3}H]nicotinic acid labels the site that is responsible for G protein activation. The difference in potencies between [^{3}H]nicotinic acid binding (K_d 22.8 nM) and [^{35}S]GTPγS binding studies (EC_{50} 1.42 μM) may be caused by the different incubation condi-

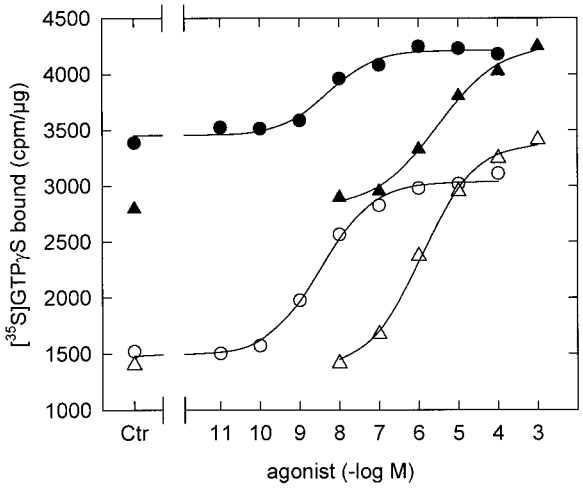


Fig. 4. Stimulation of G protein activation in epididymal adipocyte membranes by nicotinic acid and CCPA. Nicotinic acid (△, ▲) and CCPA (○, ●) dose response curves were measured in the absence (open symbols) or presence (closed symbols) of maximally stimulating concentrations of the second agonist. Incubations were done with 1 μg of protein/tube for 90 min at 25°C as described under *Experimental Procedures*. Data from one representative experiment of three are shown. EC_{50} values determined for nicotinic acid were 1.20 μM under control conditions (95% confidence limits: 0.695–2.07 μM) and 2.52 μM (1.72–3.68 μM) in the presence of 10 μM CCPA (▲). EC_{50} values of CCPA were 4.05 nM under control conditions (1.80–9.09 nM) and 3.98 nM (1.62–9.74 nM) in the presence of 1 mM nicotinic acid (●).

TABLE 1

Stimulation of [^{35}S]GTPγS binding to rat adipocyte and spleen membranes

G protein activation by nicotinic acid and related heterocycles was measured with 1 μg of membrane protein from epididymal adipocytes or spleen as described under *Experimental Procedures.* EC_{50} values are geometric means with 95% confidence limits from 3 to 10 independent experiments.

Compound	EC_{50}	
	Adipocytes	Spleen
	μM	
Nicotinic acid	1.42 (1.20–1.68)	0.703 (0.604–0.817)
Pyridazine-4-carboxylic acid	3.76 (2.71–5.21)	3.14 (2.12–4.66)
Acipimox	10.3 (6.52–16.2)	6.56 (5.85–7.35)
3-Pyridine-acetic acid	16.4 (14.3–18.7)	17.1 (10.8–26.9)
Pyrazine-2-carboxylic acid	26.2 (21.9–31.3)	21.8 (15.4–31.0)
5-Methylnicotinic acid	30.2 (20.1–45.6)	30.0 (13.1–68.7)
5-Methylpyrazine-2-carboxylic acid	52.0 (32.7–82.9)	14.5 (8.36–25.0)
6-Methylnicotinic acid	72.6 (51.5–103)	53.7 (26.7–108)
Nicotinic acid-1-oxide	80.4 (58.5–110)	73.7 (55.2–98.4)
2-Hydroxynicotinic acid	132 (93.8–186)	N.D.
Furane-3-carboxylic acid	142 (101–198)	N.D.
Nicotinamide	>1000	>1000

N.D., not determined.

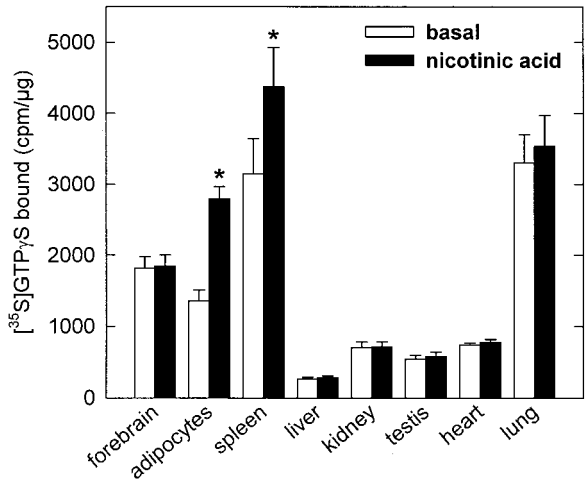


Fig. 5. G protein activation by nicotinic acid in membranes from rat tissues. [^{35}S]GTPγS binding to membranes from various rat tissues (1–2 μg/tube) in the absence (open columns) and in the presence (filled columns) of 1 mM nicotinic acid was measured after 90 min of incubation at 25°C. Results are means ± S.E.M. from three experiments. *Significant ($p < 0.05$) difference between basal and nicotinic acid-stimulated binding of [^{35}S]GTPγS.

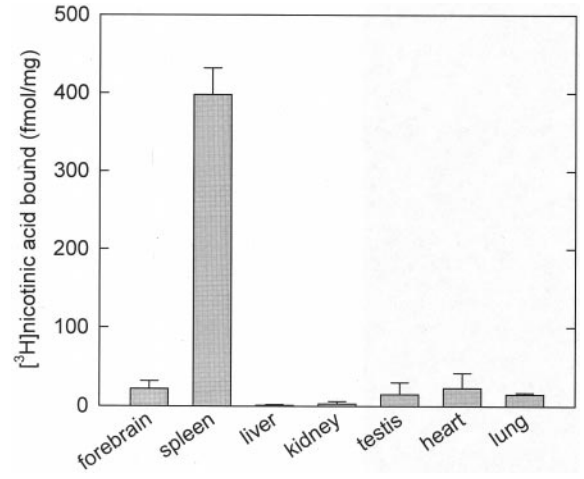


Fig. 6. Binding of [^{3}H]nicotinic acid to membranes from rat tissues. Specific binding of [^{3}H]nicotinic acid to membranes (100 μg/tube) from various rat tissues was measured after 3.5 h of incubation at 25°C. Specific binding was determined as the difference between total binding of [^{3}H]nicotinic acid (20 nM) and the nonspecific binding in the presence of 100 μM acipimox. Results are means ± S.E.M. from three independent experiments.

tions in the different assays. Sodium ions and GDP were present in the G protein activation assay, but not in [^{3}H]nicotinic acid binding experiments. The potency of nicotinic acid in [^{35}S]GTPγS binding was approximately 6-fold higher in the absence of sodium chloride [247 (174–350) nM]. In the presence of 100 mM NaCl, [^{3}H]nicotinic acid binding was reduced to 77.3 ± 0.4% of control levels (three experiments). Likewise, when the assay was performed in the presence of 100 mM sodium chloride and 1 μM GDP instead of 10 μM, an EC_{50} value of 237 (122–460) nM was determined.

It has been reported previously that GTPase activation and adenylyl cyclase inhibition by nicotinic acid in adipocyte membranes are pertussis toxin-sensitive (Aktories et al., 1983). In agreement with these findings, we found that pertussis toxin pretreatment of rat adipocyte membranes reduced stimulation of [^{35}S]GTPγS binding to 24.5 ± 7.6% of control membranes (four experiments). In contrast, [^{3}H]nicotinic acid binding to spleen membranes was reduced only to 84.3 ± 5.6% by pertussis toxin.

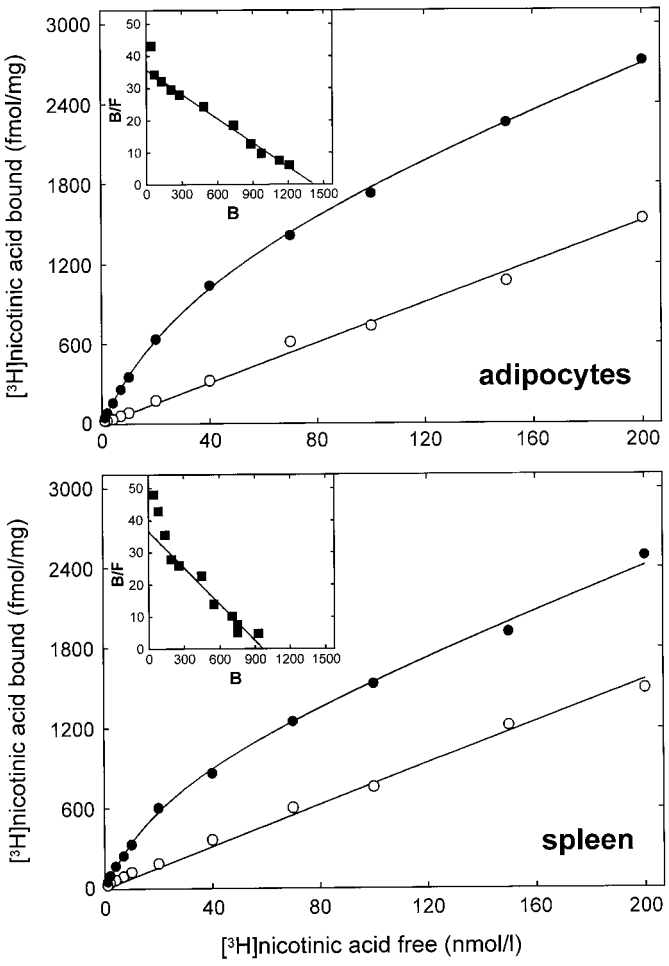


Fig. 7. Saturation of binding sites with [^{3}H]nicotinic acid in rat epididymal adipocyte and spleen membranes. Increasing concentrations of [^{3}H]nicotinic acid were incubated with adipocyte membranes (50 μg/tube; upper panel) or spleen membranes (75 μg/tube; lower panel) for 3.5 h at 25°C. ●, total binding; ○, nonspecific binding in the presence of 100 μM acipimox. The insets display the Scatchard plots of the data. One of three independent experiments for each membrane type is shown.

Discussion

Nicotinic acid and related heterocyclic agents activate pertussis toxin-sensitive GTPase and inhibit adenylyl cyclase in a hormone-like manner (Aktories et al., 1980a,b, 1982, 1983). A specific receptor has been proposed (Aktories et al., 1980a), but no direct evidence for its existence has been presented so far. In the present study, we have used a different approach to characterize the effects of nicotinic acid on G proteins. EC_{50} values for nicotinic acid (~1 μM) and acipimox (~10 μM) in [^{35}S]GTPγS binding experiments were in good agreement with EC_{50} values of these compounds in GTPase activation and adenylyl cyclase inhibition (Aktories et al.,

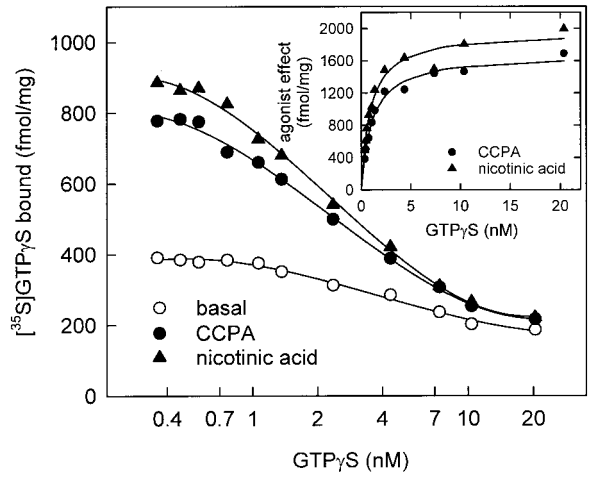


Fig. 8. Saturation analysis of agonist-induced G protein activation in rat epididymal adipocyte membranes. Saturation analysis was performed using homologous displacement of [^{35}S]GTPγS with unlabeled GTPγS. [^{35}S]GTPγS binding was carried out as described under *Experimental Procedures* with 2 μg/tube membrane protein in the absence (○) or presence of 10 μM CCPA (●) or 100 μM nicotinic acid (▲). The inset shows the agonist-induced increases in GTPγS binding as transformed to obtain saturation binding isotherms. Representative curves from one experiment are shown. Similar results were obtained in two additional experiments.

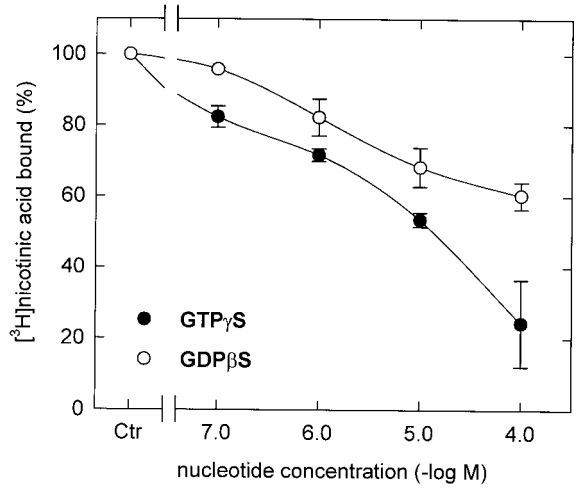


Fig. 9. Inhibition of [^{3}H]nicotinic acid binding by guanine nucleotides. [^{3}H]Nicotinic acid (20 nM) binding under control conditions (no nucleotide) and in the presence of increasing concentrations of GDPβS (○) or GTPγS (●) to rat spleen membranes (100 μg/tube) is shown as percentage of specific binding. Nonspecific binding was measured in the presence of 100 μM acipimox. Total binding under control conditions was 1991 ± 192 cpm/tube. Results are given as means ± S.E.M. from three independent experiments.

1980b), indicating that the three different approaches characterize the same transduction pathway. In agreement with a previous study on adenylyl cyclase inhibition (Aktories et al., 1980c), nicotinamide was inactive in G protein activation determined as stimulation of [^{35}S]GTPγS binding (Table 1).

We investigated whether nicotinic acid might activate known G protein-coupled receptors. However, the site through which nicotinic acid induces G protein activation is distinct from other G_i/G_o-coupled receptors, which have been detected previously in adipocyte or spleen membranes. Binding sites for neuropeptide Y or peptide YY are detectable in adipocyte membranes from human, dog, and mouse fat cells, but not in membranes from rat adipocytes (Castan et al., 1994). The α_2-adrenergic agonist 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK 14,304) did not stimulate [^{35}S]GTPγS binding in adipocyte or spleen membranes (data not shown), in agreement with the finding that UK 14,304 inhibits lipolysis in adipocytes from old rats (at least 12 weeks old and ~340 g of weight), but not in younger animals (Gasic and Green, 1995), as used in the present study (6–8 weeks, ~150 g). Glucagon-like peptide-1 (1–36) amide and glucagon-like peptide-1 (7–36) amide inhibit isoproterenol-stimulated lipolysis and decrease cyclic AMP levels in 3T3-L1 adipocytes through a receptor distinct from the pancreatic receptor for glucagon-like peptide (Montrose-Rafizadeh et al., 1997). In rat adipocyte membranes, glucagon-like peptide-1 (1–36) amide did not stimulate [^{35}S]GTPγS binding (not shown), which excludes the possibility that nicotinic acid might act on receptors for glucagon-like peptide-1. Membrane receptors for angiotensin II (subtype 1) have been characterized previously in rat epididymal adipocyte membranes (Crandall et al., 1993). However, angiotensin II did not mimic the stimulatory effect of nicotinic acid in G protein activation, and the angiotensin receptor antagonist [Sar^1,Ile^8]angiotensin II did not affect the stimulation by nicotinic acid (data not shown). Somatostatin binding sites have been identified in rat adipocytes (Simón et al., 1988). All five somatostatin receptor subtypes inhibit adenylyl cyclase via G proteins (Hoyer et al., 1994), and their mRNAs are also detectable in rat spleen (Bruno et al., 1993). The analog somatostatin (1–14), which is a potent and nonselective agonist at all somatostatin receptor subtypes (Hoyer et al., 1994), did not stimulate [^{35}S]GTPγS binding to adipocyte membranes (not shown). Therefore we conclude that the nicotinic acid effect is independent from somatostatin receptors. Nonselective agonists of muscarinic receptors (carbachol) and cannabinoid receptors [*R*(+)-WIN 55,212-2 mesylate] were also inactive (data not shown).

TABLE 2

Inhibition of [^{3}H]nicotinic acid binding to rat spleen membranes

Inhibition of [^{3}H]nicotinic acid (20 nM) binding to rat spleen membranes by nicotinic acid and structurally related compounds was assessed in the presence of 100 μg of membrane protein after 3.5 h of incubation at 25°C, as described under *Experimental Procedures*. K_i values were determined by nonlinear curve fitting and are given as geometric means with 95% confidence limits from at least three independent experiments.

Inhibitor	K_i
	nM
Nicotinic acid	33.0 (31.5–34.6)
Pyridazine-4-carboxylic acid	121 (81.7–179)
Acipimox	309 (263–362)
3-Pyridine-acetic acid	403 (370–439)
5-Methylpyrazine-2-carboxylic acid	652 (594–716)
5-Methylnicotinic acid	717 (456–1130)
Pyrazine-2-carboxylic acid	762 (618–940)
6-Methylnicotinic acid	1700 (1370–2120)
Nicotinic acid-1-oxide	3160 (2810–3550)

The actions of nicotinic acid were compared with the effects of CCPA, which is an agonist of the G protein-coupled A_1 adenosine receptor. Like CCPA, the stimulatory effect of nicotinic acid required the presence of micromolar concentrations of GDP (Fig. 3). A_1 adenosine receptors and the putative nicotinic acid receptor are present in rat epididymal adipocyte membranes in approximately the same densities. When A_1 receptors were labeled with the agonist radioligand [^{3}H]*R*-N^6-phenylisopropyladenosine (*R*-PIA), a binding density of 1890 fmol/mg of membrane protein has been measured (Trost and Schwabe, 1981). The density of [^{3}H]nicotinic acid binding sites determined in the present study (1518 ± 115 fmol/mg) is in the same range as that for A_1 receptors in fat cell membranes. The A_1-selective agonist CCPA and nicotinic acid induce a similar magnitude of G protein activation above basal levels (Fig. 8). CCPA activated 1482 fmol/mg of G protein, and nicotinic acid activated 1755 fmol/mg. Receptor densities determined in saturation experiments indicate that A_1 receptor agonists and nicotinic acid activate approximately one G protein per A_1 adenosine receptor or per [^{3}H]nicotinic acid binding site. The efficacy in signal transduction between these two receptors is therefore very similar.

Although CCPA and nicotinic acid are similarly effective in activation of pertussis toxin-sensitive G proteins in adipocyte membranes, it has been shown in long-term treatments of rat adipocytes with A_1 receptor agonists or nicotinic acid that the effects of these agents are not equal. Prolonged incubation of adipocytes with A_1 adenosine receptor agonists decreases the density of A_1 receptors, leads to a decrease in the content of G protein α_{i1}-, α_{i2}-, α_{i3}-, and β-subunits and attenuates the antilipolytic response to A_1 agonist as well as to insulin (Green, 1987; Green et al., 1990, 1997). In contrast, desensitization with nicotinic acid diminishes the sensitivity of adipocytes to nicotinic acid, but not to insulin, and down-regulation of G proteins is not observed (Green, 1987; Green et al., 1997). Heterologous desensitization via down-regulation of G_i is induced by prolonged incubation of adipocytes in the presence of the A_1 adenosine receptor agonist *R*-PIA (Green et al., 1992). Pretreatment with *R*-PIA diminishes the antilipolytic effects of *R*-PIA, but also of PGE_1 and, to a smaller degree, that of nicotinic acid. In contrast, nicotinic acid pretreatment, possibly due to the absence of G_i down-regulation, exclusively diminishes the responsiveness to nicotinic acid, but not to *R*-PIA or prostaglandin E_1, pointing to a fundamental difference in the regulation of G proteins by A_1 receptor agonists and nicotinic acid. In line with these observations in desensitization studies, we have found that activation of G proteins by the A_1-selective agonist CCPA and nicotinic acid are almost additive, without mutual effects of these agonists on the potency of the second agonist (Fig. 4). This finding does not necessarily imply that A_1 receptors and the nicotinic acid receptors are coupled to distinct G protein subtypes. G_{i2} is the most important transducer of hormonal inhibition of adenylate cyclase by *R*-PIA and nicotinic acid in adipocytes, with G_{i1} or G_{i3} capable of the same action, but with lower efficacy (Rudolph et al., 1996). The G protein pools activated by A_1 adenosine receptors and nicotinic acid may belong to functionally compartmentalized G protein pools and are therefore subject to different modes of regulation.

Binding sites for [^{3}H]nicotinic acid were identified in membranes from adipocytes and spleen (Fig. 7), the same tissue membranes in which nicotinic acid-induced G protein activation had been observed (Fig. 5). The finding that nicotinic acid significantly affected G protein activity in the spleen and the presence of specific binding sites in spleen for this hypolipidemic drug was unexpected because the spleen is generally not viewed as a relevant target organ for nicotinic acid. Future studies could help to clarify the relative importance of nicotinic acid effects in spleen. No binding of [^{3}H]nicotinic acid was detected in membranes from other rat tissues (Fig. 6). The rank orders of potencies in G protein activation and inhibition of [^{3}H]nicotinic acid binding were identical, indicating that the site labeled by [^{3}H]nicotinic acid is the site which is responsible for G protein activation. All nicotinic acid-related heterocyclic compounds shown to activate G proteins in rat adipocyte or spleen membranes stimulated [^{35}S]GTPγS binding to the same levels as nicotinic acid and are therefore considered full agonists. Agents that did not enhance [^{35}S]GTPγS binding also did not inhibit the stimulatory effects of nicotinic acid. Likewise, all competition curves from [^{3}H]nicotinic acid binding inhibition experiments with structurally related binding inhibitors were monophasic. These results therefore indicate that all active compounds were agonists. [^{3}H]Nicotinic acid binding was inhibited by GTPγS more potently than by GDPβS (Fig. 9), as expected for agonist binding to the high-affinity state of G protein-coupled receptors. However, the Scatchard plots of [^{3}H]nicotinic acid saturation experiments in spleen membranes were slightly curvilinear, which might indicate that the radioligand could label two sites of different affinities. Although fitting the saturation data to a two-site model did not improve the fit significantly, we have addressed this possibility by inhibition of [^{3}H]nicotinic acid binding by unlabeled nicotinic acid in the absence and presence of 10 and 100 μM GTPγS. In the presence of GTPγS, lower maximum binding capacities were determined, as expected from inhibition experiments shown in Fig. 9. GTPγS also reduced the affinity of nicotinic acid approximately 2-fold. Since the inhibition curves in the absence as well as in the presence of GTPγS were strictly monophasic, the GTPγS-induced decrease in affinity cannot be attributed to a shift of nicotinic acid receptors from the high- to the low-affinity state. Alternatively, if the binding affinities of this receptor in the G protein-coupled and uncoupled state are not very different, these results do not exclude the possibility that [^{3}H]nicotinic acid labels both affinity states. To clarify this question, a radioligand antagonist would be required, which is currently not available.

In agreement with a previous report (Aktories et al., 1983), we have found that G protein activation by nicotinic acid in adipocyte membranes is sensitive to pertussis toxin. However, [^{3}H]nicotinic acid to spleen membranes was inhibited by the toxin only to a minor extent. This may indicate that the majority of G proteins coupled to the nicotinic acid receptor in spleen are distinct from G_i/G_o. On the other hand, [^{3}H]nicotinic acid may bind to G protein-coupled and uncoupled receptors with similar affinities. The relative insensitivity to pertussis toxin in spleen membranes may be due to the possibility that the majority of receptors are uncoupled. This assumption is in agreement with the low efficacies of guanine nucleotides on [^{3}H]nicotinic acid binding. Again, to clearly discriminate between these two possibilities, an antagonist radioligand of the nicotinic acid receptor would be required.

To conclude, the results support the concept that the effects of nicotinic acid are transduced via a specific G protein-coupled receptor located in membranes of fat and spleen cells. Because it provides a better signal-to-noise ratio and requires less membrane protein, stimulation of [^{35}S]GTPγS binding to adipocyte membranes by nicotinic acid and analogs is a more advantageous screening method than GTPase and adenylate cyclase studies. Its site of action was characterized for the first time in direct receptor binding studies. The finding that nicotinic acid receptors are present not only in adipocytes but also in spleen may prove to be relevant in understanding the therapeutic effects of this compound. Characterizing the functional effects of nicotinic acid on specific cell types in spleen will be an important issue in further studies. A more detailed functional exploration of the nicotinic acid receptor will be possible when antagonists become available. An important target will be the elucidation of the amino acid sequence and structural properties of this receptor.

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