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Nicotinic Acid Receptor Agonists Differentially Activate Downstream Effectors*

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Nicotinic acid remains the most effective therapeutic agent for the treatment and prevention of atherosclerosis resulting from low high density lipoprotein cholesterol. The therapeutic actions of nicotinic acid are mediated by GPR109A, a G_i protein-coupled receptor, expressed primarily on adipocytes, Langerhans cells, and macrophage. Unfortunately, a severe, cutaneous flushing side effect limits its use and patient compliance. The mechanism of high density lipoprotein elevation is not clearly established but assumed to be influenced by an inhibition of lipolysis in the adipose. The flushing side effect appears to be mediated by the release of prostaglandin D2 from Langerhans cells in the skin. We hypothesized that the signal transduction pathways mediating the anti-lipolytic and prostaglandin D2/flushing pathways are distinct and that agonists may be identified that are capable of selectively eliciting the therapeutic, anti-lipolytic pathway while avoiding the activation of the parallel flush-inducing pathway. We have identified a number of GPR109A pyrazole agonists that are capable of fully inhibiting lipolysis *in vitro* and *in vivo* and not only fail to elicit a flushing response but can antagonize the ability of nicotinic acid to elicit a flush response *in vivo*. In contrast to flushing agonists, exposure of cells expressing GPR109A to the non-flushing agonists fails to induce internalization of the receptor or to activate ERK 1/2 mitogen-activated protein kinase phosphorylation.

Nicotinic acid (niacin, vitamin B3, pyridine-3-carboxylic acid) is the most effective therapeutic agent to date for raising high density lipoprotein (HDL)[2] levels. It also offers protection against other cardiovascular risk factors by lowering very low density lipoprotein (VLDL), low density lipoprotein (LDL), and lipoprotein(a) plasma concentrations (1, 2). Although the mechanism by which nicotinic acid raises HDL is not clear, one hypothesis is that it is the ability of nicotinic acid to inhibit lipolysis in adipocytes that results in a decrease in the concentration of free fatty acids available for the liver to use for triglyceride synthesis and VLDL production. The attenuated synthesis of the triglyceride-rich VLDL particles in the liver leads to a decreased rate of HDL metabolism via limiting the cholesterol ester transfer protein (CETP)-mediated exchange of cholesterol from HDL to VLDL, and triglyceride from VLDL to HDL (3–6). Another hypothesis is that nicotinic acid inhibits the uptake and subsequent catabolism of Apo-AI-containing HDL particles in hepatocytes (7, 8).

Identification of a high affinity nicotinic acid binding site that was localized to adipose, macrophage, and spleen tissues and appeared to function in a G_i protein-coupled manner (9) led to the molecular identification of the high affinity nicotinic acid receptor GPR109A (HM74A in humans and PUMA-G in mice) (10–12). In the adipose, GPR109A mediates an anti-lipolytic response that can attenuate cAMP-stimulated lipolysis (11). A low affinity nicotinic acid receptor has also been identified, referred to as GPR109B or HM74 (11). GPR109B appears to be the product of the gene duplication of GPR109A and is >95% identical to GPR109A. A search of available genomes indicates that this receptor is found only in the human and chimpanzee genomes and is absent in rodents. It is therefore difficult to know whether GPR109B has an endogenous ligand or plays a physiological role.

The therapeutic value of nicotinic acid is limited by its major side effect, cutaneous flushing. This burning sensation, felt on the face and upper body, is responsible for a large portion of patient non-compliance (13–15). Recent work has begun to elucidate the mechanism by which nicotinic acid induces flushing (16–19). GPR109A has been shown to mediate nicotinic acid-induced flushing through release of prostaglandin D2 (PGD_2) and involves the activation of the DP1 receptor and possibly a PGE_2 receptor (EP2 or EP4) (16, 18) Recent work has further supported the hypothesis that it is GPR109A receptors on Langerhans cells in the skin that mediate nicotinic acid-induced flushing through generation of PGD_2 (17, 19).

A series of pyrazole derivatives have been reported in the literature that act as partial agonists for the nicotinic acid receptor (20). The authors postulate that tissue selectivity, commonly observed with partial agonists, could be useful in pre-

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[2] The abbreviations used are: HDL, high density lipoprotein; MAP, mitogen-activated protein; NEFA, non-esterified fatty acid; PGD_2, prostaglandin D2; VLDL, very low density lipoprotein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase.

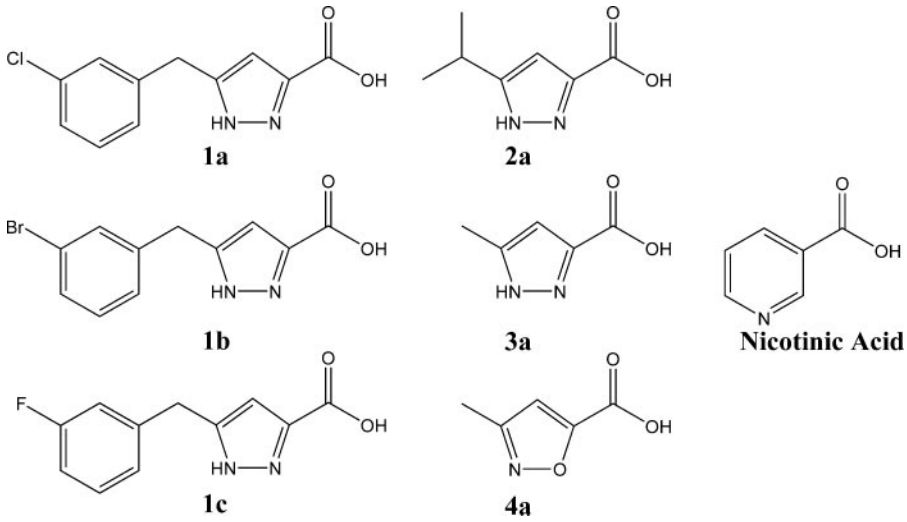

FIGURE 1. **Structures of GPR109A agonists presented herein with compound designations indicated below each structure.**

venting unwanted effects on skin cells and thus reduce or eliminate flushing. In the work presented herein, we set out to test the hypothesis that there are GPR109A agonists that are effective anti-lipolytic agents that do not cause flushing. We find that we can divide GPR109A agonists into two groups, those that elicit an anti-lipolytic and a flushing response, exemplified by nicotinic acid, and those that only elicit an anti-lipolytic response, *i.e.* non-flushing agonists. This second group of non-flushing compounds significantly decreases plasma free fatty acids and not only fails to elicit a flushing response but can also block the flushing response of nicotinic acid. The non-flushing agonists are comprised of both full and partial agonists, depending on the functional output examined. We further characterized the signaling pathways elicited by these compounds and have shown that compounds that led to a flushing response also induce both ERK 1/2 mitogen-activated protein (MAP) kinase activation as well as receptor internalization. The non-flushing compounds fail to significantly activate ERK 1/2 MAP kinase and do not result in receptor internalization. These non-flushing agonists may have a therapeutic, anti-lipolytic benefit without the unwanted cutaneous flushing side effect.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Generation of Stable CHO-K1-GPR109A Cells

The GPR109A receptor was cloned and stable CHO-K1 cells were generated as described in Ref. 21.

Animal Use and Protocols

Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (1996). All study protocols were reviewed and approved by Arena Pharmaceutical's Internal Animal Care and Use Committee. C57Bl/6 mice were purchased from Jackson Laboratory, and jugular vein-catheterized Sprague-Dawley rats were purchased from Charles River Laboratories.

Measurement of Adenylyl Cyclase Inhibition

A 96-well Adenylyl Cyclase Activation Flashplate Assay™ kit (PerkinElmer) was optimized to measure changes in intracellular cAMP levels due to receptor activation in CHO-K1 stable cell lines expressing GPR109A. CHO-K1 cells were cultured in F-12 Kaighn's modified cell culture medium with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 400 μg/ml Geneticin in T-185 cell culture flasks. Cells were harvested from culture flasks and isolated via low speed centrifugation, counted, and diluted to a density of 1×10^6 cells/ml. Compounds were prepared in Me_2SO and serially diluted with phosphate-buffered saline (PBS). Cells were added to the test plates containing the compounds at a final cell density of 50,000 cells/well. 5 μM forskolin was added to all wells. The assay plates were placed on a shaker for 1 h at room temperature (~25 °C). Diluted ^{125}I-cAMP (supplied with the FlashPlate kit) was added to each well and the plates continued to shake for another 2 h. The plates were counted on a Wallac Microbeta Counter 1450.

Human Subcutaneous Fat Lipolysis Assay

Preparation of Adipocytes—Cultured human subcutaneous adipocytes were received from Zen Bio., Inc. plated in 96-well plates 2 weeks prior to performing the lipolysis assay. Upon arrival, all medium was removed and pooled together (ZenBio Adipocyte Maintenance medium). 150 μl of this medium was then re-aliquoted to each well. Cells were kept in a sterile, humidified incubator at 37 °C and allowed to recover for 1 week.

Induction of Lipolysis—On the day of the lipolysis assay, cells were washed twice with 150 μl of Zen Bio wash buffer. After the second wash and removal of wash buffer, 75 μl of test compounds were added to each well, in triplicate. Compounds were prepared in Zen Bio assay buffer plus 1 μM isoproterenol. Cells were incubated for 5 h at 37 °C.

Glycerol Assay—Glycerol was determined using a free glycerol reagent from Sigma (Reagent A). Adipocyte medium (50 μl) was removed and transferred to a flat-bottom 96-well plate. Reagent A (50 μl) was then added to each well. After 15 min, absorbance was read at A_{540} on a Spectramax 340PC microplate reader (Molecular Devices). The amount of glycerol released was calculated based on regression analysis of known glycerol concentrations using a glycerol standard (Sigma).

Mouse Non-esterified Fatty Acid (NEFA) Assay

Male C57/Bl6 mice, 8–10 weeks old (~25 g), were food-deprived 16 h prior to the experiment. They were then injected intraperitoneal with vehicle (PBS) or the compound of interest at the desired concentration (5 ml/kg) diluted in PBS. After 10

		IC_{50}	Relative Inhibition	N
△	**1a**	4.2 μM	65%	10
●	**1b**	1.7 μM	67%	2
▲	**1c**	2.2 μM	96%	8
◇	**2a**	2.6 μM	98%	4
○	**3a**	434 nM	100%	12
✳	**4a**	1.3 μM	100%	12
■	**NA**	67.3 nM	100%	9

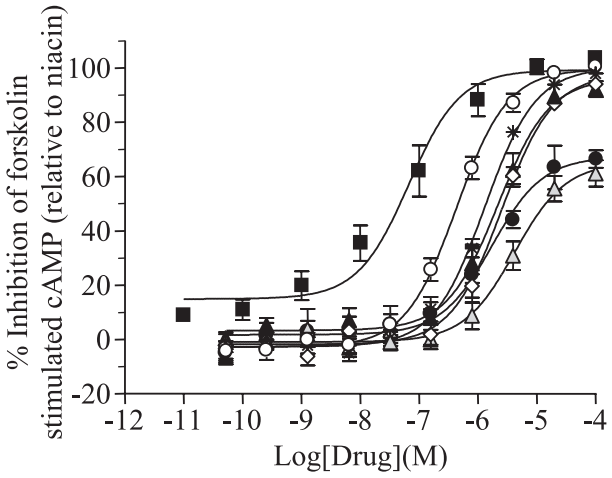

FIGURE 2. **Inhibition of forskolin-stimulated cAMP in stably transfected CHO-K1 cells.** GPR109A agonists were added at the indicated concentrations to measure their ability to inhibit forskolin (5 μM)-stimulated cAMP production. Nicotinic acid was used as the full agonist, positive control. Values represent mean % inhibition of forskolin-stimulated cAMP production normalized to nicotinic acid ± S.E. IC_{50} values are shown in the table above. Height represents the % maximal inhibition of forskolin-stimulated cAMP relative to nicotinic acid.

min, the animals were euthanized via CO_2 asphyxiation and blood was drawn by cardiac puncture. The blood was centrifuged on a tabletop centrifuge (4000 rpm at 4 °C for 10 min). Serum was collected in a new microfuge tube, recentrifuged (4000 rpm at 4 °C for 10 min), and dispensed to a 96-well plate at 5 μl/well for NEFA assay. The NEFA assay was done according to the manufacturer's suggested protocol (NEFA-C kit; Wako) adding 100 μl of reagent A, incubating for 10 min at 37 °C, adding 200 μl of reagent B, incubating for another 10 min at 37 °C, and equilibrating for 5 min at room temperature (~25 °C). Absorbance was measured on a plate reader at 550 nm.

Rat NEFA Assay

Jugular vein cannulae of male Sprague-Dawley rats (7–10 weeks old; ~350 g) were flushed with ~200 μl of saline, and animals were food-deprived for 16 h. All blood draws were immediately followed by a 200-μl saline flush to maintain patency of the cannulae. Base-line blood samples were collected via cannula, and rats were given vehicle (PBS, 5% hydroxypropyl-β-cyclodextrin, or 0.5% methylcellulose) or drug via per oral administration (2 ml/kg). Blood was collected at desired time points, transferred to a microcentrifuge tube, and placed on ice. The blood was centrifuged on a tabletop centrifuge

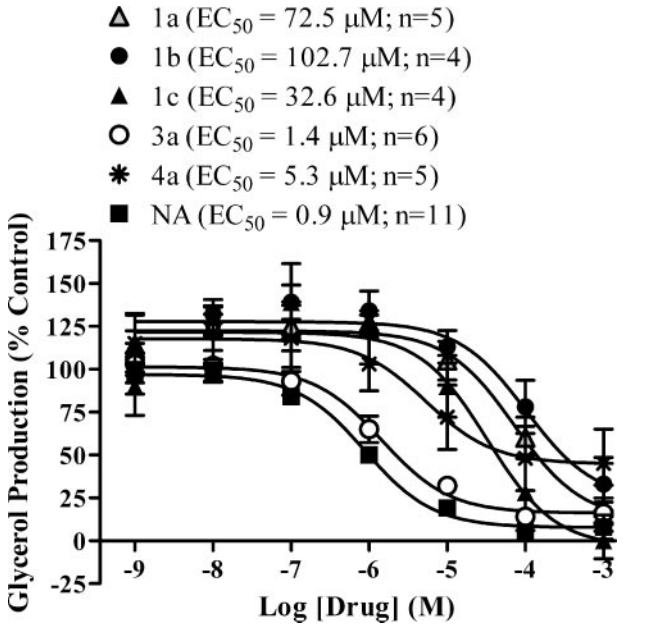

FIGURE 3. **Human subcutaneous adipose *in vitro* lipolysis.** Human subcutaneous adipocytes were treated with 1 μM isoproterenol to stimulate lipolysis and then exposed to agonists at the indicated concentrations. Values are the mean % of control (isoproterenol-stimulated) ± S.E. of four or more experiments, each run in triplicate (EC_{50} and replicate values are indicated in the *key at the top* (*n*)).

(4000 rpm at 4 °C for 10 min). Serum was collected in a new microfuge tube and processed as described for the mouse NEFA assay above.

Mouse Flushing via Laser Doppler

Male C57/Bl6 mice (8–10 weeks old; ~25 g) were anesthetized with Nembutal via intraperitoneal injection (80 mg/10 ml/kg). After 10 min the mouse was placed under an LDPI Laser Doppler (PeriScan PIM II; Perimed, Stockholm) and a needle and syringe containing vehicle (PBS, 40% hydroxypropyl-β-cyclodextrin, or 0.5% methylcellulose) or drug was placed in the intraperitoneal space and a slight back pressure was applied to prevent premature delivery of compound. The right ear of the mouse was turned inside-out to expose the ventral side using forceps. The Laser Doppler was focused in the center of the ventral right ear and adjusted as follows: repeated data collection; 15 × 15 image format; auto interval start; 20-s delay; medium resolution; very fast scan speed; and 8–9-V intensity (~4.5 cm from ear). After a 3-min base-line reading, vehicle or compound was administered into the intraperitoneal space (5 ml/kg through the preinserted syringe), and readings continued for ~15 min.

For competition assays, a similar protocol was utilized with the exception that immediately following the Nembutal injection the mouse was intraperitoneal-injected with vehicle or competing compound. This was followed by establishing base-line and intraperitoneal drug administration (typically 30 mg/kg nicotinic acid) as described above. In some cases, this round of flushing was followed by re-establishing base-line and intraperitoneal PGD_2 (2 mg/5 ml/kg) injection as a positive control.

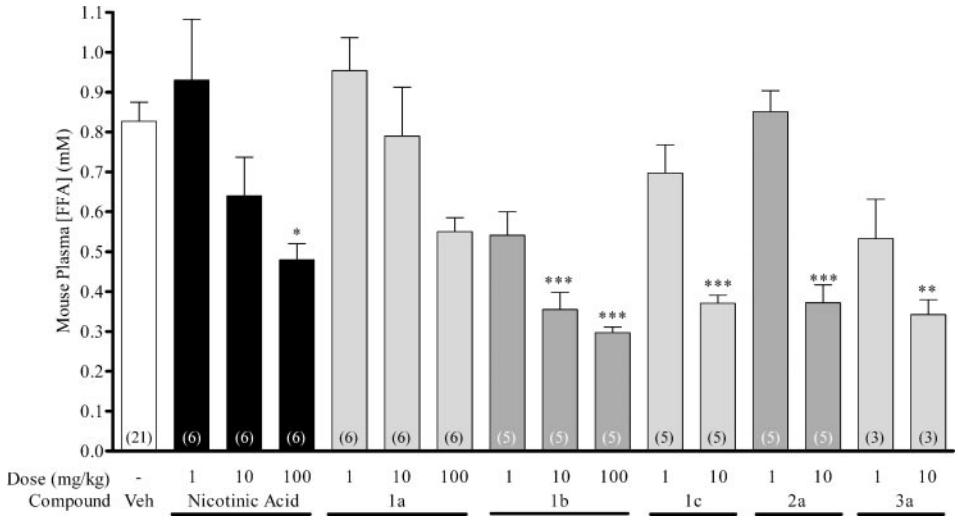

FIGURE 4. **Mouse *in vivo* lipolysis (NEFA) assays.** Male C57Bl/6 mice were per oral-injected with vehicle (PBS) or compounds as indicated, and blood was collected 10 min post-treatment. Values are the mean plasma FFA concentration (mM) ± S.E. of three or more experiments each run in triplicate as indicated (*n*). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ via one-way analysis of variance with Bonferroni's multiple comparison post-hoc test.

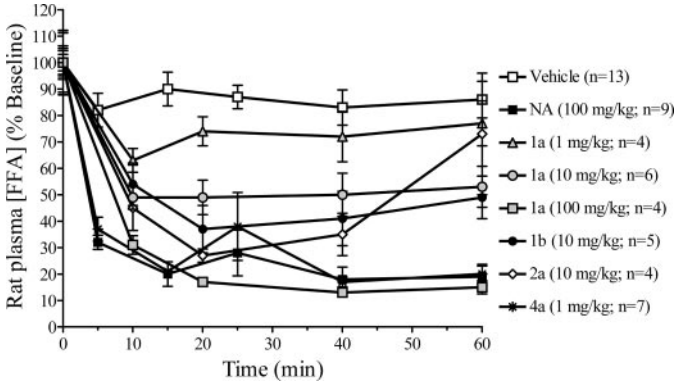

FIGURE 5. **Rat *in vivo* lipolysis (NEFA) assays.** Male Sprague-Dawley rats were per oral-injected with vehicle (PBS) or compounds as indicated, and blood was collected via jugular vein catheter at the indicated time points post-treatment. Values are the mean % base-line plasma FFA concentration ± S.E. of four or more independent experiments as indicated (*n*).

MAP Kinase Assays

MAP kinase assays were performed using the phospho-MAP kinase enzyme-linked immunosorbent assay kit (KHO 0091) from BIOSOURCE according to the manufacturer's specifications. Specifically, CHO-K1 stable cell lines expressing GPR109A were serum-starved overnight. Cells were stimulated with compounds for 5 min at 37 °C, the medium was aspirated, and the cells were rinsed with PBS. The cells were scraped in 1 ml of PBS and transferred to a microfuge tube. The suspension was centrifuged for 5 min at 3000 rpm, and the pellet was resuspended in 200 μl of cell extraction buffer (0.1% SDS). The samples were incubated on ice for 30 min and then clarified by centrifugation for 10 min, 4 °C at 13,000 rpm. Protein concentrations were determined by a modified Bradford analysis, and 10 μg of protein was added to each well of a 96-well plate coated with anti-phospho-MAP kinase capture antibody. The samples were incubated for 2 h at 25 °C and then extensively washed before incubation with the anti-phospho-MAP kinase detection antibody for 1 h at 25 °C. The samples were washed and then incubated with an horseradish peroxidase-conjugated secondary antibody for 30 min at 25 °C. The samples were washed and then incubated with chromogen in the dark for 20 min at 25 °C before stopping the reaction with stop buffer. Absorbance at 450 nm was read on a Spectramax 340PC microplate reader (Molecular Devices).

For the mouse ear MAP kinase assays, mice were injected intraperitoneal with either vehicle or niacin at 100 mg/kg. After 5 min, mice were sacrificed via CO_2 asphyxiation and the ears removed. Ears were minced into small pieces and homogenized in lysis buffer using a Brinkman Polytron. Membrane protein was isolated via centrifugation at 20,000 rpm (JA-25.50 rotor, 15 min, 4 °C). Membrane pellets were resuspended in 200 μl of cell extraction buffer and treated as described above.

Detection of GPR109A Internalization

COS-7 cells were transiently co-transfected with NH_2-terminal HA epitope-tagged human or mouse GPR109A and human β-arrestin2 and plated on glass coverslips in 24-well plates (1×10^5 cells/well). Receptor internalization was assayed at 48 h after transfection. Cells were washed three times with PBS and incubated on ice for 1 h with Alexa 488-conjugated anti-HA-antibody 16B12 (Molecular Probes) at 5 μg/ml in Opti-MEM. Indicated concentrations of compounds were then added to the cells and incubated at 37 °C for various lengths of time. Cells were washed three times with PBS and fixed with formaldehyde. Coverslips were then mounted on slides for visualization.

Mouse Mixed Epidermal Cell Culture

Mouse epidermal cells were isolated and cultured as described in Ref. 19.

PGD_2 Enzyme-linked Immunosorbent Assays

The PGD_2 content of culture medium was assayed as described in Ref. 19 using 2×10^6 cells/ml. For the assay, 50 μl of cell suspension was added to 400 μl of prewarmed (37 °C) RPMI containing 20 μM MEK inhibitor PD98059 or vehicle (Me_2SO) and incubated at 37 °C, 5% CO_2 for 15 min. 50 μl of RPMI containing 1 mM nicotinic acid, 10 μM indomethacin, or vehicle were added to each well and the cells were further incubated for 30 min and treated as described in Ref. 19.

RESULTS

Compounds used in the assays described herein are shown in Fig. 1. CHO-K1 cells were stably transfected with GPR109A and cellular cAMP was measured in the intact cells. Compounds were added at the indicated concentrations to measure their ability to inhibit 5 μM forskolin-stimulated cAMP levels, and nicotinic acid was used as the full agonist positive control. 5-Methyl-3-carboxyl-pyrazole (*3a*), 3-methyl-5-carboxyl-isox-

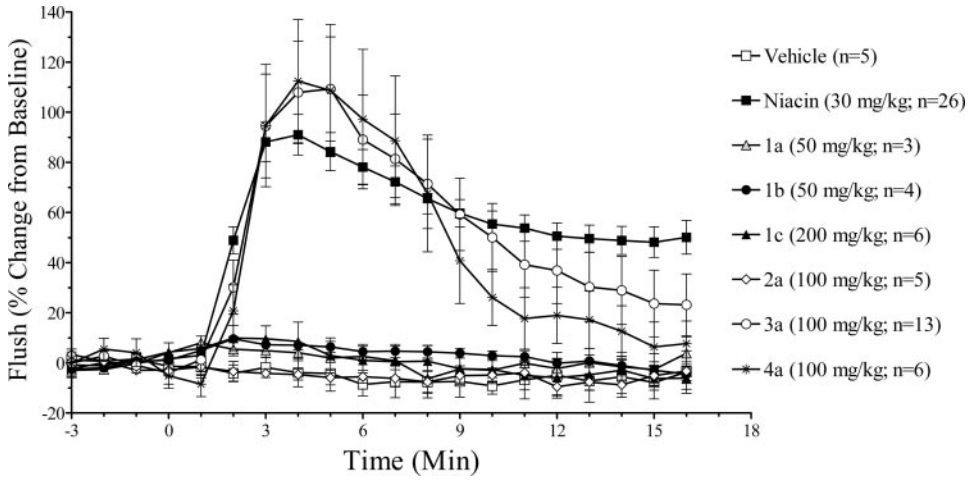

FIGURE 6. **Mouse flushing via Laser Doppler.** Base-line perfusion was established for 3 min in the ventral right ear of C57Bl/6 mice. Mice were then given intraperitoneal injections of individual GPR109A agonists. Values are the mean % change from base-line, preinjection values ± S.E. of 3–26 individual animals as indicated in the *key at right*.

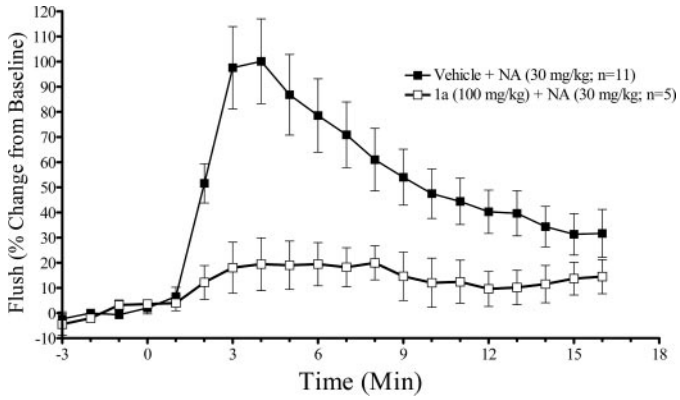

FIGURE 7. **Competition of nicotinic acid-induced flush in mice.** C57Bl/6 mice were given intraperitoneal injections of vehicle or compound 1a 10 min prior to determining base line (co-administered with the Nembutal anesthetization). Base line was established followed by intraperitoneal injection of nicotinic acid. Values are the mean % change from base-line, prenicotinic acid injection values ± S.E. of eleven or five animals as indicated in the *key at right*. Preinjection of compound 1a antagonized the ability of nicotinic acid to induce a flushing response.

azole (*4a*), 5-isopropyl-3-carboxyl-pyrazole (*2a*), and 5-meta-fluorobenzyl-3-carboxyl-pyrazole (*1c*) inhibited forskolin-stimulated cAMP production to the same extent as nicotinic acid (*i.e.* were full agonists; Fig. 2). By contrast, 5-meta-chlorobenzyl-3-carboxyl-pyrazole (*1a*) and 5-meta-bromobenzyl-3-carboxyl-pyrazole (*1b*) were able to inhibit only 65–67% of the forskolin-stimulated cAMP production (*i.e.* were partial agonists; Fig. 2). These compounds had the same potency and efficacy when applied to cAMP assays using cells stably transfected with human, rat, or mouse GPR109A. The partial agonists 1a and 1b were applied to [^{3}H]nicotinic acid competition binding assays and were able to fully displace [^{3}H]nicotinic acid binding (data not shown).

We examined the ability of these compounds to inhibit human adipocyte lipolysis in an *in vitro* model. Human subcutaneous adipocytes were cultured and differentiated as described under "Experimental Procedures." Lipolysis was stimulated with 1 μM isoproterenol, and compounds were added to determine their ability to inhibit lipolysis. Fig. 3 shows the results of the adipocyte lipolysis assays. All of the compounds tested inhibited isoproterenol-stimulated lipolysis with varying potencies. All but one compound, 4a, appeared to inhibit lipolysis to the same extent as nicotinic acid (*i.e.* full inhibition; Fig. 3). Compound 4a was only able to decrease glycerol production to 50% of the control value (Fig. 3), indicating that it is a partial agonist in this assay.

We next examined the effect of these GPR109A agonists on plasma free fatty acid concentrations *in vivo* in male C57Bl/6 mice. Again, all of the compounds examined were able to acutely depress plasma free fatty acid concentrations to an equivalent or greater extent than nicotinic acid 10 min after per oral agonist administration (Fig. 4). This effect appeared to be dose-dependent for each of the agonists examined. The potency of the compounds varied but did not necessarily reflect the predicted, *in vitro*, potency of the agonists. This more likely reflects the bioavailability and/or compound half-life.

When we applied the GPR109A agonists to an acute, *in vivo* rat model of plasma free fatty acid measurement, we found similar results. All of the compounds examined dose and time dependently depressed plasma free fatty acids levels (Fig. 5). The maximal extent of plasma free fatty acid inhibition was similar for all of the agonists (up to ~75% inhibition of base-line values).

The cutaneous flushing induced by these agonists was assessed via Laser Doppler (which measures blood flow, and thus vasodilation) on the ears of male C57Bl/6 mice. Fig. 6 illustrates that some of the GPR109A agonists described above led to a flushing response (*nicotinic acid*, *3a*, and *4a*) whereas others did not (Fig. 6, *1a*, *1b*, *1c*, and *2a*). When compound 1a, a non-flushing GPR109A agonist, was preinjected into the mice 10 min prior to base line (13 min prior to nicotinic acid injection), nicotinic acid was unable to elicit a flushing response (Fig. 7). When similar experiments were performed with a follow-up PGD_2 injection, the animals responded with a concomitant full flushing response (data not shown). These data indicate that the non-flushing compound 1a is able to interact with receptors on the cells responsible for mediating the flushing response and antagonizes the nicotinic acid-mediated flushing effect (Fig. 7).

Anti-phospho ERK 1/2 MAP kinase enzyme-linked immunosorbent assays were performed on the GPR109A stably transfected CHO cells. Interestingly, of the compounds tested only the flush-inducing compounds nicotinic acid, 5-methyl-3-carboxyl-pyrazole (compound 3a), and 3-methyl-5-carboxyl-isoxazole (compound 4a) stimulated MAP kinase activation (Fig. 8). On the other hand, the non-flush-inducing compounds 5-meta-chlorobenzyl-3-carboxyl-pyrazole (1a) and 5-isopro-

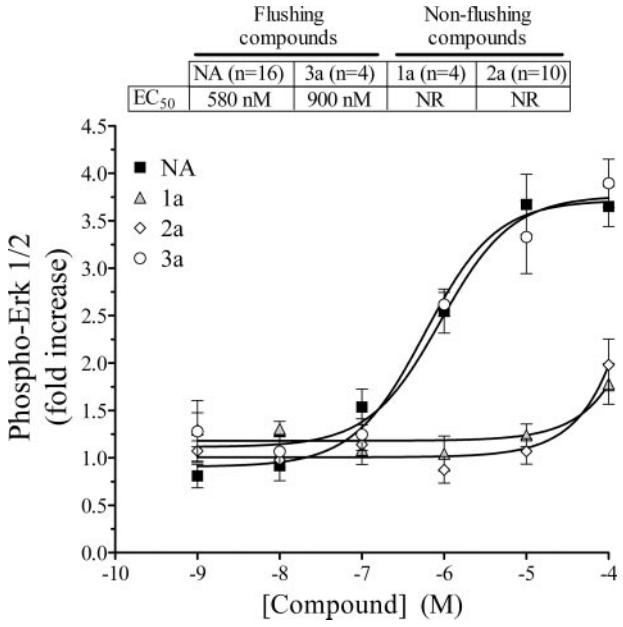

FIGURE 8. **Presence or absence of ERK 1/2 MAP kinase stimulation in stably transfected CHO-K1 cells.** Values are the mean –fold change relative to control ± S.E. The *Table inset* above summarizes the EC_{50} and experimental number (*n*) for ERK 1/2 MAP kinase activation for the compounds tested. *NR* indicates no response for which an appropriate EC_{50} could be calculated (*i.e.* goodness of fit $R^2 < 0.4$).

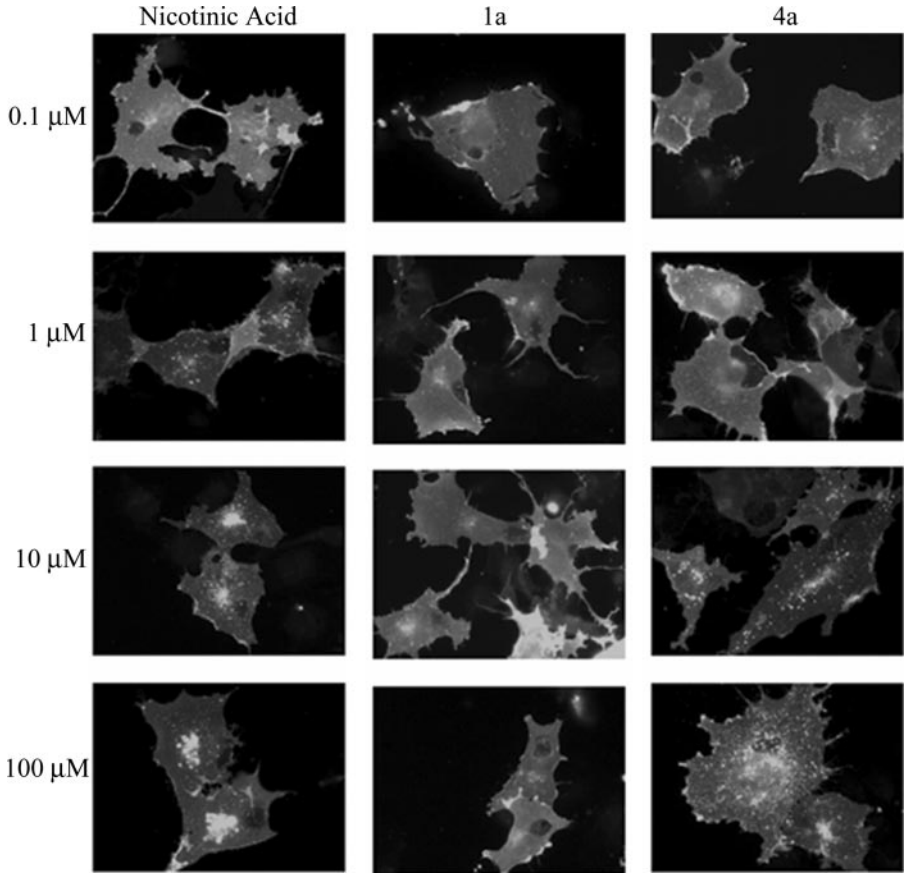

FIGURE 9. **Qualitative assessment of GPR109A internalization following agonist exposure.** The dose-dependent internalization of GPR109A was examined using immunofluorescence microscopy on transiently transfected COS-7 cells following 30 min of exposure to the indicated doses of nicotinic acid (*left*), compound 1a (*middle*), or compound 4a (*right*). Data shown are representative of at least three independent experiments.

pyl-3-carboxyl-pyrazole (2a) failed to significantly increase ERK 1/2 MAP kinase activity (Fig. 8).

It was postulated that β-arrestin recruitment and receptor internalization are required for MAP kinase activation (as proposed by Daaka *et al.*) (22). To test this, we examined the ability of these compounds to differentially internalize epitope-tagged GPR109A in transiently transfected COS-7 cells. Fig. 9 shows the dose-dependent effects of nicotinic acid and 5-meta-chlorobenzyl-3-carboxyl-pyrazole (compound 1a) on the cellular localization of HA-tagged GPR109A. Following 30 min of exposure of the cells to 1, 10, and 100 μM nicotinic acid or the isoxazole agonist 4a, anti-HA immunoreactivity appears to cluster in a characteristic pattern within the subcellular space (Fig. 9, *left* and *right rows*), indicative of agonist-directed receptor internalization. On the other hand, compound 1a, a non-flushing agonist, failed to effect any change in anti-HA immunoreactivity, confirming the hypothesis that this agonist fails to drive receptor internalization (*middle row*). When applied to other GPR109A agonists described herein, the qualitative assessment of HA-tagged GPR109A immunoreactivity revealed that, again, nicotinic acid stimulation results in receptor internalization, whereas non-flushing compounds 2a and 1a– c fail to internalize receptor immunoreactivity (Fig. 10).

Recent work examining the mechanism of niacin-induced flushing has revealed that it is mediated by GPR109A (16) and requires the release of PGD_2 and activation of DP1 receptors in the skin (18) and, finally, that the PGD_2 release is mediated by epidermal Langerhans cells (17, 19). Based on the results shown herein we would speculate that GPR109A agonists activate a MAP kinase-mediated release of PGD_2 from Langerhans cells, leading to the cutaneous vasodilation responsible for the flushing response. As shown in Fig. 11*A*, mice given an intraperitoneal niacin injection had a profound, 3-fold elevation of phospho-MAP kinase in the ear (where flushing is measured in the mouse model). When mouse epidermal cells were isolated and cultured, niacin treatment led to a significant PDG_2 release that was attenuated by pretreatment with the MEK inhibitor PD98059 (Fig. 11*B*). This effect was also seen in cultured human Langerhans cells where both the MEK inhibitor and indomethacin (a cyclooxygenase inhibitor) were able to attenuate PGD_2 release (Fig. 11*C*). Although *in vivo* use of the MEK inhibitor to prevent flushing was confounded by its inability to inhibit MAP kinase production in

this model, the above results are consistent with a MAP kinase requirement for flushing.

DISCUSSION

The results presented herein indicate that there are GPR109A agonists that, like nicotinic acid, are capable of stimulating the G_i-mediated inhibition of adenylyl cyclase, inhibit isoproterenol-stimulated lipolysis in adipocytes, and decrease plasma free fatty acids in both mice and rats but that lack the ability to induce receptor internalization and ERK 1/2 MAP kinase activation. We find that the compounds that fall into this group fail to elicit a flushing response and, in fact, can antagonize the flushing response elicited by nicotinic acid. These results suggest that it may be possible to tailor anti-lipolytic compounds that fail to induce the unwanted flushing side effect present in nicotinic acid preparations.

The concept that a receptor agonist may selectively activate only a subset of signal effectors distinct from other agonists via a common receptor has been proposed for a number of G protein-coupled receptors. This phenomenon may result if the effectors being examined are sequential and the agonists used have differing intrinsic efficacies resulting in differential signal strength. One agonist may induce a first effector sufficient to overcome a threshold necessary to activate a second, downstream effector signal, whereas another agonist is capable of eliciting a measurable, first effector signal that is not sufficient to activate the second effector pathway.

Alternatively, it has been proposed that receptors such as G protein-coupled receptors may, upon activation, have more than one

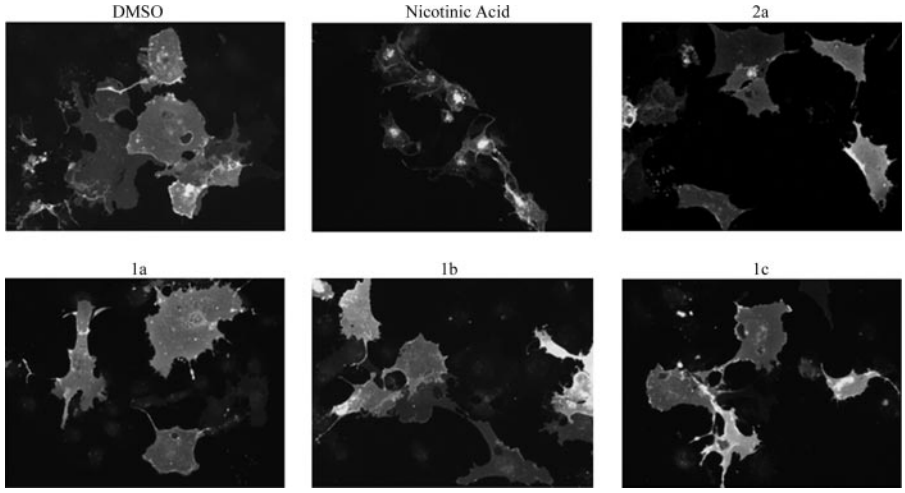

FIGURE 10. **Qualitative assessment of GPR109A internalization following 10 μM agonist exposure for 30 min.** Internalization of GPR109A was examined using immunofluorescence microscopy on transiently transfected COS-7 cells following 30 min of exposure to 10 μM of indicated compounds. Data shown are representative of at least three independent experiments.

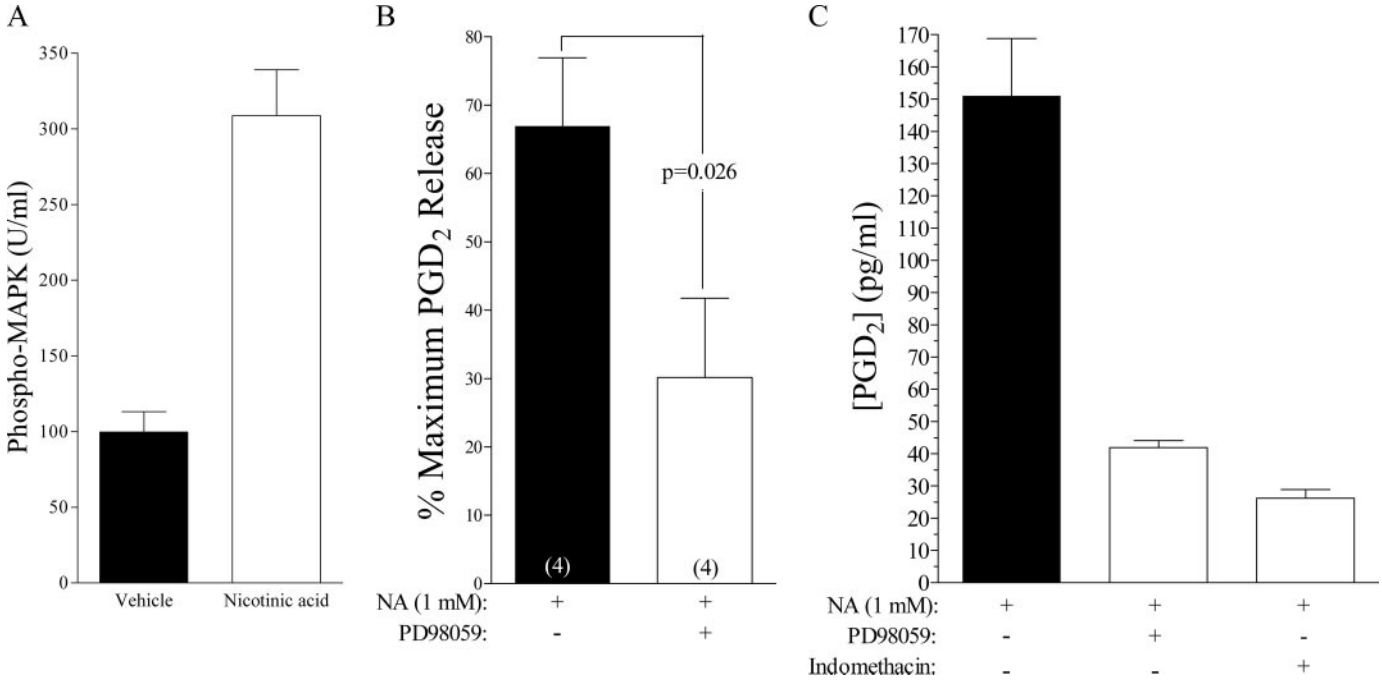

FIGURE 11. **Niacin-mediated MAP kinase and PGD_2 production in mouse skin and human Langerhans cells.** *A*, niacin mediates a >3-fold stimulation of phospho-ERK 1/2 MAP kinase in the ears of mice intraperitoneal-injected with 100 mg/kg niacin relative to vehicle-treated mice. Data shown are the mean ± S.D. of values derived in triplicate from two mice. *B*, mouse epidermal cells were isolated and cultured. Cells were incubated for 15 min with 20 μM PD98059 or Me_2SO vehicle control and then treated with 1 mM niacin for 30 min. Niacin-mediated PGD_2 released into the culture medium was measured, basal PGD_2 values were subtracted out as background, and values were normalized to the maximal PGD_2 release elicited in the individual experiments. Values are the mean ± S.E. of four independent experiments. *p* value determined by Student's *t* test. *C*, human Langerhans cells were incubated for 15 min with 20 μM PD98059, indomethacin, or Me_2SO vehicle control and then treated with 1 mM niacin for 30 min. Niacin-mediated PGD_2 release was attenuated by pretreatment with either PD98059 or indomethacin.

active conformation. In this model one agonist would induce a conformation capable of activating multiple, parallel effector pathways, whereas another agonist induces a receptor conformation only capable of eliciting a subset of signaling effectors. This model of pleiotropic agonist signaling has been termed "agonist-directed trafficking of receptor signals" (23).

The idea that different agonists may induce different receptor conformations or may have different signal strengths, and therefore divergent signaling pathways, has been suggested by a number of groups for a variety of G protein-coupled receptors including the α_{2A}-adrenergic receptor, 5HT2 receptors, adenosine-A1, and the nicotinic acid receptor (Refs. 20, 24–28 and reviewed by T. Kenakin, Ref. 23). In these studies it is suggested that use of one agonist or agonist class may elicit cellular and pharmacological activities distinct from activities elicited by another agonist or agonist class.

These concepts have been adopted by a number of groups for the use of partial agonists, compounds that bind receptors but in which activation does not translate to a fully efficacious response, to elicit a desired response without an undesirable side effect. For example, it was shown by Tan *et al.* (24) that use of an α_{2A}-adrenergic receptor partial agonist, moxonidine, has an anti-hypertensive effect in wild-type C57Bl/6 mice while avoiding the sedative side effect elicited by the α_{2A}AR full agonist clonidine. In this study it is important to note that different agonists elicit *in vitro* responses with varying degrees of efficacy as a function of the different effectors examined (*e.g.* GTPγS binding, MAP kinase activation, etc.). In addition, the receptor density and downstream effectors are going to vary in different tissues and therefore the relative efficacies of agonists will also vary accordingly. The authors conclude that partial agonists can evoke response-selective pathways and may be used to provide response-selective therapies (24).

As described in the Introduction, a series of pyrazole derivatives have been reported in the literature that act as partial agonists for [^{35}S]GTPγS binding via the nicotinic acid receptor in rat adipocyte and spleen membranes (20). The authors postulate that tissue selectivity, commonly observed with partial agonists, could be useful in preventing unwanted, flush-inducing side effects on skin cells while maintaining the ability to inhibit lipolysis.

In examination of the compounds presented herein, when we looked at agonist effects at inhibiting cAMP we identified four full agonists (compounds 1c and 2–4a) and two partial agonists (compounds 1a and 1b); of these, three are non-flushing compounds (one full agonist and both of the partial agonists). These effects are independent of the species of the receptor as the potency and efficacy of these compounds were essentially identical in human, rat, or mouse stably transfected cells. In the *in vitro* lipolysis assay one of the compounds, 4a, is a partial agonist, yet this compound is a potent flush inducer. It should be noted that these compounds have little or no affinity to GPR109B, the low affinity niacin receptor. In addition, as mentioned in the Introduction, GPR109B does not appear to have a rodent ortholog. Therefore, differential effects mediated by the panel of molecules presented herein cannot be explained on the basis of non-selectivity.

Therefore, the separation of downstream effector signals in order to achieve a therapeutic effect without the unwanted side effect does not necessarily require the development of partial agonists. Rather, the drug design and identification process should be focused on identification of agonists that potently stimulate responses downstream of effectors known to mediate therapeutic responses and that lack the ability to stimulate effectors that mediate untoward side effects. Obviously, because the mechanism of therapeutic action of nicotinic acid has yet to be identified, one concern would be that eliminating the side effect may also eliminate the desired therapeutic effect. Preclinical and clinical studies need to be done comparing the therapeutic efficacy of compounds that fall into the two categories, those that elicit flushing, MAP kinase activation, and internalization in addition to eliciting an anti-lipolytic response *versus* those that solely elicit an anti-lipolytic response.

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