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# The ancient drug salicylate directly activates AMP-activated protein kinase

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## Abstract

Salicylate, a plant product, has been in medicinal use since ancient times. More recently it has been replaced by synthetic derivatives such as aspirin and salsalate, both rapidly broken down to salicylate *in vivo*. At concentrations reached in plasma following administration of salsalate, or aspirin at high doses, salicylate activates adenosine monophosphate-activated protein kinase (AMPK), a central regulator of cell growth and metabolism. Salicylate binds at the same site as the synthetic activator, A-769662, to cause allosteric activation and inhibition of dephosphorylation of the activating phosphorylation site, Thr172. In AMPK knockout mice, effects of salicylate to increase fat utilization and lower plasma fatty acids *in vivo* were lost. Our results suggest that AMPK activation could explain some beneficial effects of salsalate and aspirin in humans.

The medicinal effects of willow bark have been known since the time of Hippocrates. The active component is salicylate, a hormone produced by plants in response to pathogen infection (1). For medicinal use it was largely replaced by aspirin (acetyl salicylate), which is rapidly broken down to salicylate *in vivo* (2, 3). Salicylate can also be administered as salsalate, which shows promise for treatment of insulin resistance and type 2 diabetes (4, 5). Aspirin and salicylate inhibit cyclo-oxygenases and hence prostanoid biosynthesis (6), as well as the protein kinase IKKβ in the NF-κB pathway (7). However, some effects of these drugs are still observed in mice deficient in these pathways (8).

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor conserved throughout eukaryotes. This heterotrimeric enzyme is composed of catalytic α

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**Experimental Procedures** Experimental procedures used in this paper are presented in a Supplementary file, as are Figures S1-S7.


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subunits and regulatory β and γ subunits (9, 10). Once activated in response to metabolic stress, AMPK phosphorylates targets that switch off adenosine triphosphate (ATP) consuming processes, while switching on catabolic pathways that generate ATP. AMPK is activated >100-fold by phosphorylation at Thr172 in the α subunit by the tumour suppressor protein kinase, LKB1, or the $Ca^{2+}$-dependent kinase, CaMKKβ (9, 10). Binding of AMP or adenosine diphosphate (ADP) to the γ subunit triggers a conformational change that promotes phosphorylation and inhibits dephosphorylation (11-15), causing a switch to the active form. Binding of AMP (but not ADP) to a second site (15) causes further allosteric activation, leading to >1,000-fold activation overall (16). Most drugs or xenobiotics that activate AMPK work by inhibiting mitochondrial ATP synthesis and increasing the concentration of AMP and ADP (17). However, a synthetic activator, A-769662 (18), which also causes allosteric activation and inhibits Thr172 dephosphorylation, binds directly to AMPK at distinct site(s) (19-21).

Salicylate, but not aspirin, activated AMPK when applied to HEK-293 cells, with its effects being significant at 1 mM and above (Fig. 1A; it appears that the esterases that catalyse breakdown of aspirin to salicylate are not expressed in these cells). This was associated with increased phosphorylation of Thr172 on AMPK-α, and the downstream target of AMPK, acetyl-CoA carboxylase (ACC); in the latter case the effects were evident at 1 mM and above (Fig. 1A). Salicylate can uncouple mitochondrial respiration (22), so we suspected that it might activate AMPK by decreasing cellular ATP and increasing AMP and ADP. To test this, we used isogenic cell lines expressing wild type AMPK (WT cells) or a mutated enzyme in which an R531G substitution in γ2 renders AMPK insensitive to AMP or ADP (RG cells) (15, 17). At concentrations <10 mM, salicylate caused similar increases in AMPK phosphorylation or activation in WT and RG cells, showing that the effect was not dependent on changes in AMP or ADP. However, at 10 mM and above there was a greater activation/phosphorylation in WT than in RG cells, suggesting that AMP- or ADP-dependent effects were also occurring at these higher concentrations (Fig. 1B). Concentrations >1 mM salicylate increased cellular oxygen uptake in both WT and RG cells. This effect was not additive with the effect of a concentration of dinitrophenol causing a maximal increase in oxygen uptake, suggesting that at these concentrations salicylate, like dinitrophenol, could dissipate the proton gradient and thus uncouple the respiratory chain from ATP synthesis (Fig. 1C). However, unlike effects of another AMPK activator ($H_2O_2$) any increases in cellular ADP:ATP ratio at salicylate concentrations below 30 mM were very small (Fig. 1D). Thus, mitochondria appear to compensate for mild uncoupling by increasing respiration. Salicylate does not activate AMPK through the $Ca^{2+}$-CaMKKβ pathway (12), because the CaMKK inhibitor STO-609 had no effect on responses to salicylate, although it blocked responses to a $Ca^{2+}$ ionophore, A23187 (Fig. S1A/S1B, supplementary data).

Using a physiological concentration of ATP in assays (2 mM), salicylate caused a 1.6-fold allosteric activation with a half-maximal effect ($A_{0.5}$) at 1.0 ± 0.2 mM (Fig. 2A). A large activation by AMP was observed at all salicylate concentrations up to 30 mM (Fig. 2B), and salicylate did not affect the concentration of AMP causing half-maximal activation ($A_{0.5}$ = 18 ± 3 or 13 ± 2 μM, with or without 10 mM salicylate, Fig. 2C). By contrast, increasing concentrations of salicylate progressively antagonized activation by 30 and 100 nM A-769662 (Fig. 2D), and 10 mM salicylate increased $A_{0.5}$ for A-769662 by >4-fold (from 39 ± 4 to 172 ± 24 nM, Fig. 2E). These results suggest that salicylate is a partial agonist acting at the same site as A-769662, causing a small activation on its own but antagonizing the larger activation by A-769662. If it binds at the same site, salicylate should protect against Thr172 dephosphorylation, as A-769662 does (19, 20). Indeed, salicylate protected AMPK (human α1β1γ1 complex) against dephosphorylation and inactivation by protein phosphatase-2Cα to the same extent as did AMP and A-769662 (Fig. 2F), although it had no

effect on PP2Cα assayed using a peptide substrate (Fig. S2). An S108A substitution in the β1 subunit abolishes effects of A-769662 on dephosphorylation, and β2-containing complexes are resistant to the drug (20, 21). As expected, an S108A substitution in the α1β1γ1 complex abolished the effects of A-769662 and salicylate, but not that of AMP (Fig. 2G), whereas there was no effect of salicylate on dephosphorylation of an α1β2γ1 complex, although there was a small effect of A-769662 (Fig. 2H). These results lend further support to the idea that salicylate binds at the same site(s) as A-769662.

To examine effects of salicylate on AMPK phosphorylation and activation in intact cells, we used HEK-293 cells carrying a Flp recombinase target site to generate isogenic lines expressing FLAG-tagged WT β1, a β1-S108A mutant, or WT β2. Using an antibody that recognizes both isoforms, endogenous β1 and β2 could be detected in the parental cells, but (as observed previously when expressing α subunits using this system (17)) these were largely replaced by FLAG-tagged β1 and β2 (with reduced electrophoretic mobility) in cells expressing recombinant β1/β2; the expression of α1, α2 and γ1 subunits was unaffected (Fig. 3A). A-769662 and salicylate caused increased activation/phosphorylation of AMPK in cells expressing WT β1 (Fig. 3B), but their effects were greatly reduced in cells expressing the β1-S108A mutant (Fig. 3C) or WT β2 (Fig. 3D), By contrast, the effects of quercetin (which acts by increasing AMP (17)) were unaffected.

To test whether salicylate has metabolic effects *in vivo* through AMPK, we used β1 knockout mice (23). Fatty acid oxidation in isolated WT hepatocytes was stimulated by salicylate or A-769662, and was associated with increased phosphorylation of AMPK and ACC (the latter regulating fat oxidation (24)). All effects were reduced or eliminated in hepatocytes from β1-KOs (Figs. 3E/F, S3). There were no changes in ADP:ATP ratios in WT cells in response to 1 to 10 mM salicylate (Fig. S4A)

We injected mice with salicylate or A-769662 at the start of a period of fasting to assess metabolism *in vivo*. The dose used generated plasma salicylate of 2.4 ± 0.4 and 2.0 ± 0.1 mM (mean ± SEM, n = 7 and 3) in WT and β1-KO mice respectively. Both agents caused phosphorylation of liver AMPK and ACC in WT but not β1-KO mice (Fig. 4A/B), although there were no changes in hepatic AMP/ATP or ADP/ATP ratios in WT mice (Fig. S4B). In WT mice, salicylate also caused phosphorylation and activation of AMPK in soleus muscle and adipose tissue (Fig. S5). Both agents depressed the respiratory exchange ratio (RER) for 6 hours after injection, consistent with a switch from carbohydrate to fat utilization; these effects were lost in β1-KOs (Fig. 4C through F). Significant depressions in RER, in WT mice only, were evident by calculating the area under the curve (Figs. S6A and S6B). When we calculated fat and carbohydrate utilization, both salicylate and A-769662 increased fat utilization in WT but not β1-KO mice (Fig. 4G); A-769662 also decreased carbohydrate utilization in WT mice (Figs. S6C and D). Both salicylate and A-769662 reduced serum non-esterified fatty acids in WT but not β1-KO mice (Fig 4H). We also studied glucose homeostasis in mice made insulin-resistant by high-fat feeding, followed by daily salicylate injections for 2 weeks. However, effects of salicylate to improve fasting glucose, fasting insulin, glucose tolerance and insulin resistance (HOMA-IR), were retained in β1-KO mice (Fig. S7), indicating that they were independent of AMPK.

Our results show that salicylate can directly activate AMPK, primarily by inhibiting Thr-172 dephosphorylation. The plasma salicylate concentrations in humans treated with oral salsalate (4) or high-dose aspirin (30-90 mg/kg) (25, 26) are 1 to 3 mM. At these concentrations, salicylate activated AMPK in WT and RG HEK-293 cells to the same extent, and did not increase cellular ADP:ATP ratios, indicating an AMP-independent mechanism. Thus, the natural product salicylate can activate AMPK via a mechanism closely related to that of A-769662, a synthetic activator derived from a high-throughput

screen (which, unlike salicylates, has poor oral availability (18)). Although the exact site(s) occupied by salicylate and A-769662 on AMPK remain unidentified, our finding that the S108A mutation abolishes activation by both agents suggests that the binding sites overlap.

Effects of salicylate on fat oxidation *in vivo* appear to require activation of AMPK-β1 complexes. Aspirin also reduces circulating lipids in obese rats and improves insulin sensitivity (7). However, in agreement with previous studies (7, 27), our results using long-term salicylate treatment of fat-fed mice (Fig. S7) indicate that effects on AMPK-independent pathways, such as IKKβ or JNK, are also important.

After oral administration, aspirin is rapidly broken down by liver, erythrocyte, and plasma esterases to salicylate (3), whose peak plasma concentrations and half-life are orders of magnitude greater than those of aspirin (2). Our findings raise the possibility that other effects of aspirin, like protective effects against development of cancer (28), may be mediated in part by AMPK. AMPK is activated by the anti-diabetic drug metformin (17, 29), and treatment of diabetics with metformin is also associated with reduced cancer incidence (30). Our results show that one thing that salicylates and metformin hold in common is their ability to activate AMPK. However, one caveat is that the doses of aspirin required to activate AMPK *in vivo* may be higher than those used in most human studies.

### One-sentence summary

Salicylate (the natural product from which aspirin is derived) is a direct activator of AMP-activated protein kinase – this novel target for salicylate drugs might explain some of their beneficial affects in humans.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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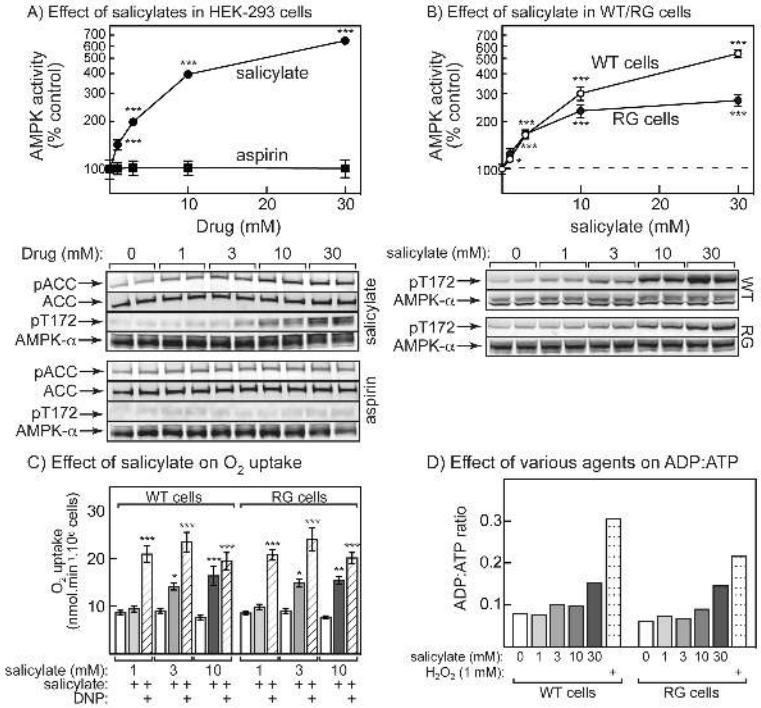


**Figure 1.**

Effects of salicylate in HEK-293 cells. (A) Effects of salicylate or aspirin on AMPK activity (mean ± SD, n = 4) and phosphorylation of AMPK (Thr172) and ACC (Ser79) (n = 2). (B) Effects of salicylate on AMPK activity (mean ± SD, n = 6) and phosphorylation (n = 2) in HEK-293 cells stably expressing wild type (WT) γ2 or an R531G substitution (RG). In Figs. 1A and 1B, the activity is plotted on a logarithmic scale as % of control without drug, and effects significantly different from control without drug (2-way ANOVA, with Bonferroni's test comparing each drug concentration to control without drug) are shown (*p<0.05, ***p<0.001). (C) Effect of salicylate on oxygen uptake in WT and RG cells (mean ± SD, n = 7 to 13; significant differences by 2-way ANOVA, using Bonferroni's test to compare with basal values without salicylate or DNP, are shown (*p<0.05, **p<0.01, ***p<0.001)). (D) Effects of salicylate or $H_2O_2$ (1 mM) on ADP:ATP ratios (means of duplicate cell incubations).




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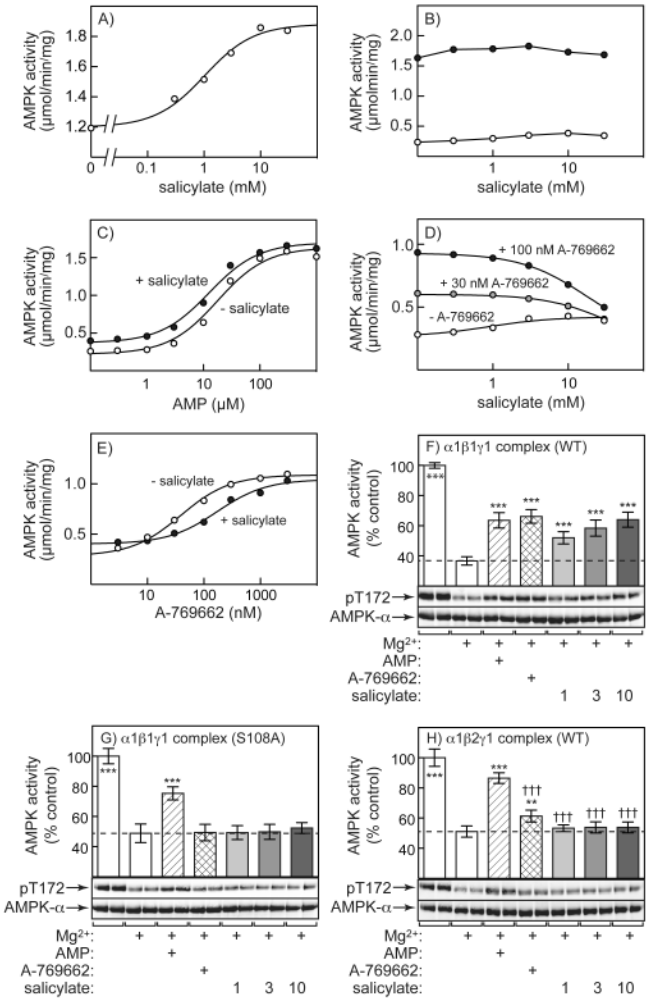


**Figure 2.**

Effect of salicylate on AMPK in cell-free assays. (A-E) Effects of salicylate on activity of purified rat liver AMPK; (A) effect of salicylate; (B) effect of salicylate ± 200 μM AMP; (C) effect of AMP ± 10 mM salicylate; (D) effect of salicylate ± 30 and 100 nM A-769662; (E) effect of A-769662 ± 10 mM salicylate. Data points in (A-E) are means of duplicate assays; lines were generated by fitting data to the equation: Y = basal + (basal*(activation - basal)*X/($A_{0.5}$ + X)); values obtained for $A_{0.5}$ and activation are quoted in the text. (F-H) Effects of AMP, A-769662 and salicylate on dephosphorylation of bacterially expressed human AMPK complexes by PP2Cα (all incubations contained PP2Cα, but control lacked $Mg^{2+}$); bar graphs show AMPK activity (% of control without $Mg^{2+}$, mean ± SD, n = 6; pictures show Thr172 phosphorylation (n = 2). (F) WT α1β1γ1 complex; (G) α1β1γ1 complex with β1 S108A substitution; (H) WT α1β2γ1 complex. Significant differences from the control plus $Mg^{2+}$, using 1-way ANOVA with Dunnett's multiple comparison test, are shown: **p<0.01, ***p<0.001. Also shown are significant differences in the size of the effect of A-76962 or salicylate between the α1β1γ1 and α1β2γ1 complexes (2F and 2H): †††p<0.001. For the latter comparisons, the difference between the "+$Mg^{2+}$+A-76922" and "+$Mg^{2+}$ only" columns was first expressed as a fraction of the difference between the "+$Mg^{2+}$+AMP" and "+$Mg^{2+}$ only" columns.

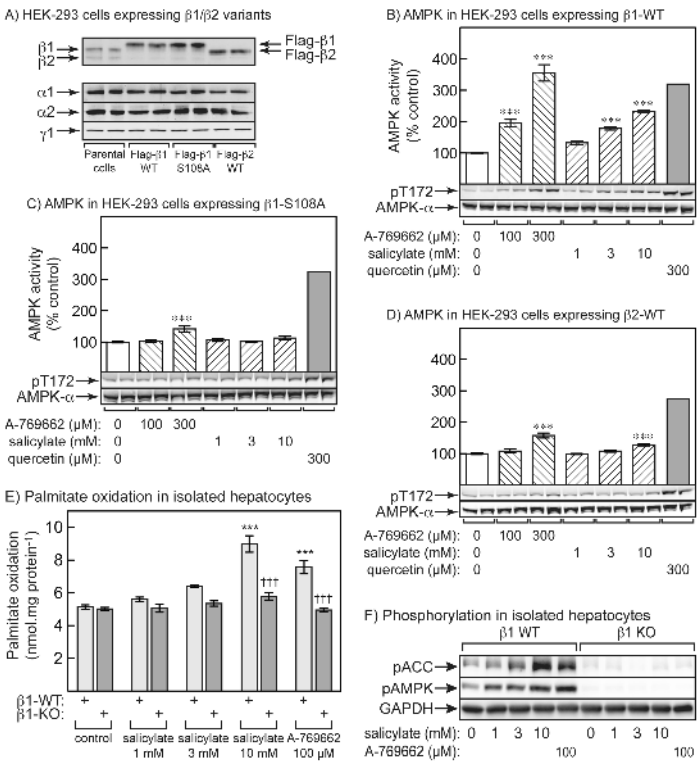


**Figure 3.**

Effects of salicylate and A-769662 in intact cells. (A) Expression of β subunits assessed using pan-β antibody in parental cells or cells stably expressing β1 WT, β1-S108A, or β2 WT, and of endogenous α1, α2 and γ1 in the same cells. (B-D) Activity and phosphorylation of AMPK after treatment with various activators in cells expressing: (B) β1 WT; (C) β1-S108A; (D) β2 WT. Kinase assays [mean ± SEM, n = 6 except for 100 μM A-769662 (n = 4) and quercetin (n = 2); significantly different from control without drug, by 1-way ANOVA with Dunnett's multiple comparison test, ***p<0.001] and Western blots (n = 2) were of immunoprecipitates made using anti-FLAG antibody. (E) Palmitate oxidation in hepatocytes isolated from β1-KO mice and WT controls (mean ± SEM, n = 6 to 14, significantly different from control without drug by 2-way ANOVA with Bonferroni's test, ***p<0.001; †††significantly different from WT, p<0.001). (F) Phosphorylation of AMPK and ACC in hepatocytes isolated from β1-KO or WT mice.




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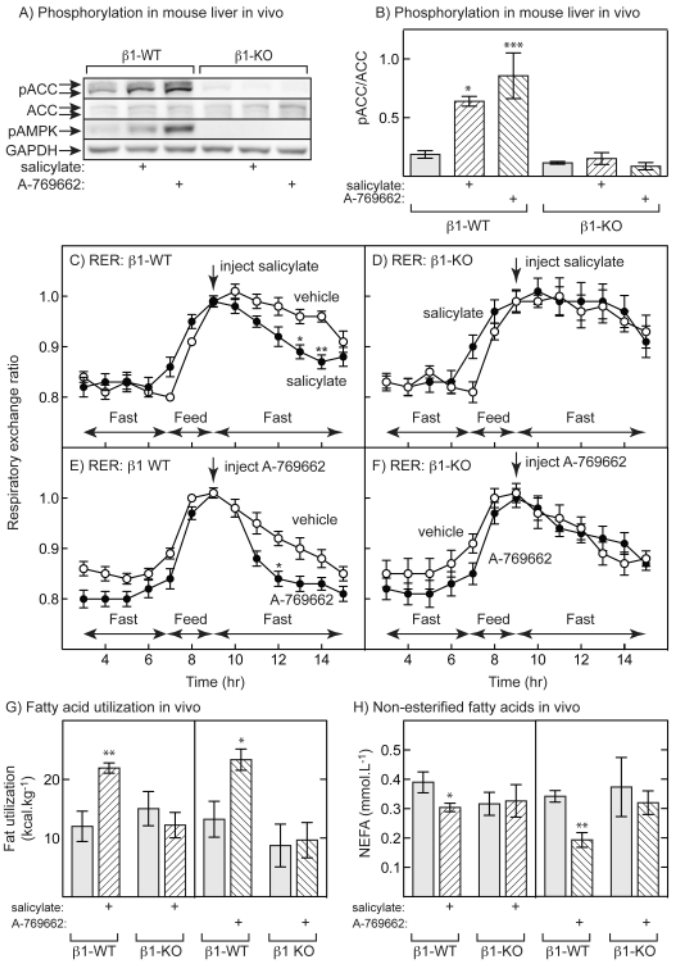


**Figure 4.**

Effects of salicylate and A-769662 treatment in β1-KO and WT mice in vivo. (A) Phosphorylation/expression of ACC, AMPK and GAPDH in liver of mice treated with salicylate or A-769662 (doublet in ACC blots represents ACC1/ACC2). (B) Quantification of phosphorylation of ACC (pACC:total ACC, mean ± SEM; n = 6 or 7 for WT, n = 3 for β1-KO; statistical significance by 1-way ANOVA with Bonferroni's test compared with vehicle only are shown (*p<0.05, ***p<0.001). (C)-(F) Respiratory exchange ratio measured in β1-KO and WT mice after injection of vehicle, salicylate (250 mg/kg) or A-769662 (30 mg/kg) at the start of a period of fasting. Results are mean ± SEM (n = 6 to 13). By 2-way ANOVA, effects of salicylate (p<0.05) or A-769662 (p<0.001) were only significant in WT mice; significant differences by Bonferroni's test at individual time points are shown (*p<0.05, **p<0.01). (G) Fatty acid utilization calculated from data in (C)-(F). Results are mean ± SEM (n = 7 or 8). Significant differences by 2-way ANOVA with Bonferroni's test are shown (*p<0.05, **p<0.01). (H) Plasma non-esterified fatty acids in mice treated with salicylate or A-769662 for 90 min. Results are mean ± SEM (n = 7 or 8). Significant differences by 2-way ANOVA with Bonferroni's test are shown (*p<0.05, **p<0.01).