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Off-Target Effects of MEK Inhibitors

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Supporting Information

ABSTRACT: The mitogen-activated protein kinases (MAPKs) ERK1/2 regulate numerous cellular processes, including gene transcription, proliferation, and differentiation. The only known substrates of the MAP2Ks MEK1/2 are ERK1/2; thus, MEK inhibitors PD98059, U0126, and PD0325901 have been important tools in determining the functions of ERK1/2. By using these inhibitors and genetically manipulating MEK, we found that ERK1/2 activation is neither sufficient nor necessary for regulated secretion of insulin from pancreatic β cells or secretion of epinephrine from chromaffin cells. We show that both PD98059 and U0126 reduce agonist-induced entry of calcium into cells in a manner independent of their ability to inhibit ERK1/2. Caution should be used when interpreting results from experiments using these compounds.

ERK1/2 play many cellular roles, including regulating glucose-induced insulin gene transcription in pancreatic β cells.[1] It is clear that many insulin secretagogues induce ERK1/2 activation.[1] As β cells secrete insulin in response to secretagogues, biosynthetic processes, including insulin gene transcription, which is dependent on ERK1/2 activation, are engaged to replenish the secreted hormone. Studies investigating the role of ERK1/2 in insulin secretion have been performed with conflicting conclusions.[2−6] Many investigators have used MEK1/2 inhibitors PD98059, U0126, and PD0325901 to investigate ERK1/2 functions.[7−10] A measure of ERK1/2 activation is the increase in the expression of the members of the Jun and Fos families that form the AP-1 transcription factor. 10 μM U0126 was sufficient to significantly reduce serum and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced c-jun and c-fos expression, while 40 μM PD98059 was needed to inhibit EGF or TPA-induced c-fos expression.[8,9] PD0325901 can inhibit the phosphorylation of downstream targets of ERK1/2 at 10 nM.[10]

We previously showed that ERK1/2 were activated 2 min after MIN6 β cells were stimulated with amino acids.[15] We observed that blockade of the ERK1/2 pathway with U0126, an inhibitor of the upstream kinases (MEK1/2), reduced the levels of amino acid-induced ERK1/2 activation and insulin secretion, suggesting that there is a component of secretion that is dependent upon ERK1/2. However, the other MEK1/2 inhibitors, PD98059 and PD0325901, did not inhibit amino acid-induced insulin secretion, despite reducing the level of ERK1/2 activation (Figure 1A,B). Because the role of ERK1/2 in insulin secretion has been in question in the literature,[2−5] we evaluated this possibility more thoroughly. To determine if prolonged activation of ERK1/2 was sufficient, we tested effects of constitutively active MEK1 on insulin secretion and found no change in secretion in spite of elevated ERK1/2 activity (Figure 1C,D). We did not observe a change in basal insulin secretion with constitutively active MEK1 (Figure 1 of the Supporting Information).

Upon further analysis of an ERK1/2 requirement for secretion, we found that two commonly used MEK1/2 inhibitors interfered with calcium homeostasis in β cells (Figure 2A). The increase in the level of intracellular free calcium induced by amino acids was strongly inhibited by PD98059 and partially prevented by U0126 (Figure 2A). A MEK1/2 inhibitor more recently available, PD0325901, even at a concentration of 500 nM had no effect on calcium changes induced by amino acids (Figure 2A). Examining the average basal free calcium prior to addition of amino acids revealed that PD98059 strongly decreased this value while U0126 slightly decreased it (Figure 2B).

We used bovine adrenal chromaffin cells stimulated with the secretagogue nicotine or depolarized with KCl to further characterize effects of PD98059. In these cells, ERK1/2 activation paralleled norepinephrine secretion very well over several minutes (Figure 2A of the Supporting Information). PD98059 blocked activation of ERK1/2 at 2 μM but did not affect secretion induced by nicotine or depolarization until the concentration exceeded 10 μM (Figure 2B of the Supporting Information and Figure 3A). Because secretion in chromaffin cells is absolutely dependent on the entry of extracellular Ca^{2+}, we examined if this process was impaired by concentrations of PD98059 that inhibit secretion. ^{45}Ca uptake was measured in cells treated with nicotine or 59 mM KCl.[11] The rate of influx of ^{45}Ca induced by nicotine or KCl was not reduced at PD98059 concentrations of <15 μM; these concentrations are sufficient to prevent ERK1/2 activation but have no effect on secretion (Figure 3B). However, at higher PD98059 concentrations, ^{45}Ca uptake was inhibited in a dose-dependent manner, reaching 80 and 50% inhibition at 75 μM PD98059 for nicotine and KCl stimulation, respectively (Figure 3B). These findings are consistent with those in β cells and suggest the generality of off-target actions of PD98059.

We reinvestigated the potential role of ERK1/2 in secretory responses and found that activation of the kinases is not sufficient for secretion from pancreatic β cells. In fact, although kinase activation parallels secretion, it is not necessary for secretion from pancreatic β cells or from adrenal chromaffin cells. Previous researchers using 100 μM PD98059 concluded

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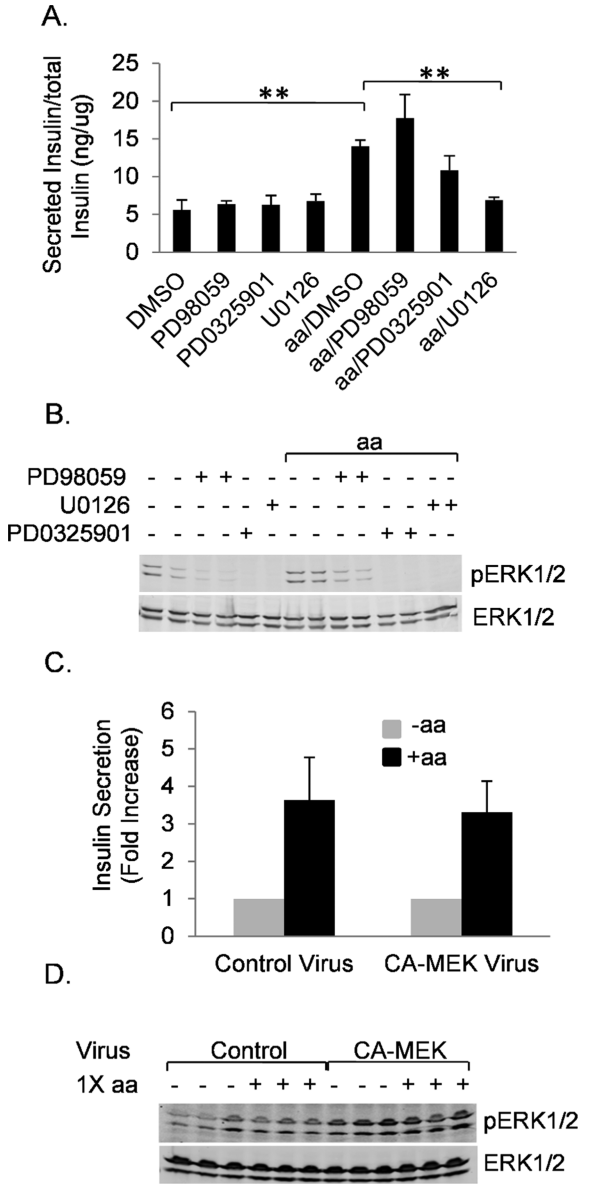

Figure 1. ERK1/2 activity is not sufficient or necessary for amino acid-induced insulin secretion. (A) MIN6 cells were incubated in Krebs-Ringer Bicarbonate Solution (KRBH) for 2 h and 45 min before being pretreated with DMSO, 20 μM PD98059, 500 nM PD0325901, or 10 μM U0126 for 15 min. Cells then were stimulated with 1× amino acids for 30 min before the KRBH was collected and the cells were lysed. The insulin content was measured in both the lysates (total insulin) and KRBH (secreted insulin) with an enzyme-linked immunosorbent assay (Supporting Information). Data are means ± the standard error of the mean (bars) representative of three independent experiments each conducted in triplicate. $^{**}p < 0.01$ (two-tailed Student's t test). (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting on the lysates from panel A. (C) MIN6 cells were infected with either a β-gal control adenovirus or a virus encoding constitutively active MEK (CA-MEK). Twenty-four hours later, cells were incubated in KRBH for 2 h and 30 min before being stimulated with 1× amino acids. Thirty minutes later, KRBH was collected, cells were lysed, and the insulin content was measured as described for panel A. The data are presented as the x-fold increase in the rate of insulin secretion induced by 1× amino acids. Data are means ± the standard deviation (bars) from two experiments each conducted in triplicate. (D) Immunoblots from the cell lysates in panel C.

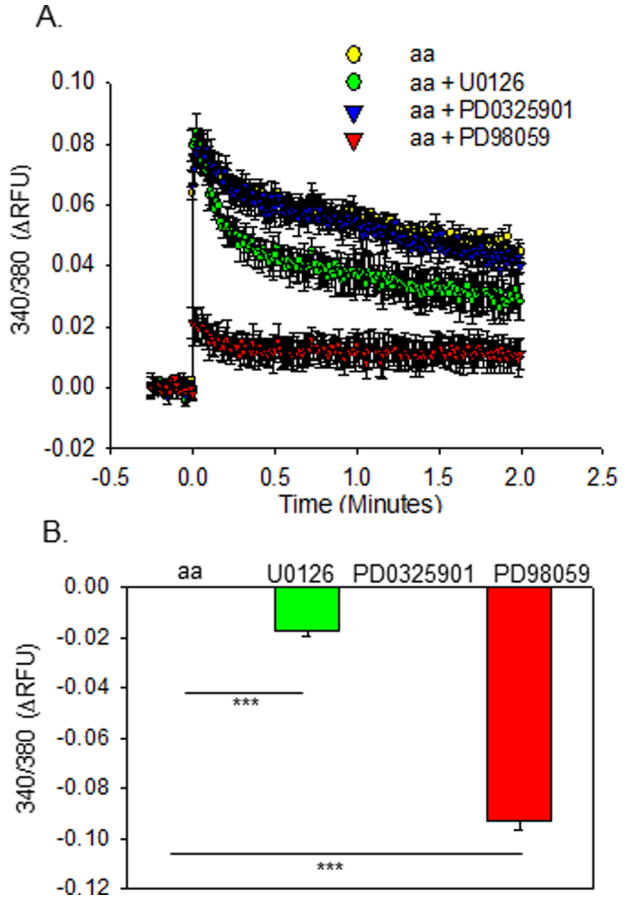

Figure 2. Uo126 and PD98059 inhibit calcium entry in a manner independent of ERK1/2 inhibition. MIN6 cells were placed in Krebs-Ringer Bicarbonate Solution without amino acids, loaded with fura-2, and pretreated with the indicated concentrations of the indicated inhibitors or DMSO (vehicle) for 30 min prior to being stimulated with amino acids. (A) Baseline ratios from each condition before the addition of amino acids were averaged and then subtracted from each of the points in the respective condition to correct for the significant differences in basal free calcium levels. (B) Baseline free calcium levels prior to amino acid stimulation. Data in both panels are means ± the standard error of the mean of the 340/380 values from three experiments, each conducted in triplicate (Supporting Information). $^{***}p < 0.001$ (two-tailed Student's t test).

that ERK1/2 activation was necessary for insulin secretion induced during the first 5 min after MIN6 cells had been stimulated with glucose.[6] However, the inhibitory effect on insulin secretion was potentially due to a decrease in calcium entry, which occurred when we used a dose of 20 μM PD98059. Reinvestigation of the effects of ERK1/2 on secretion led us to examine effects of three MEK inhibitors and discover off-target artifacts of two commonly used compounds on calcium homeostasis. Although these compounds have been of considerable value over nearly 20 years, some off-target and toxic effects of U0126 and PD98059 have been reported, generally limiting their use to short-term studies.[4,12,13] These compounds affect AMPK activity, and U0126 has recently been shown to interfere with mitochondrial respiration.[12−14] On the other hand, PD0325901 did not perturb calcium homeostasis and did not interfere with subsequent activation of ERK1/2 even after exposure for 2 days (E. Zaganjor et al., manuscript in revision).

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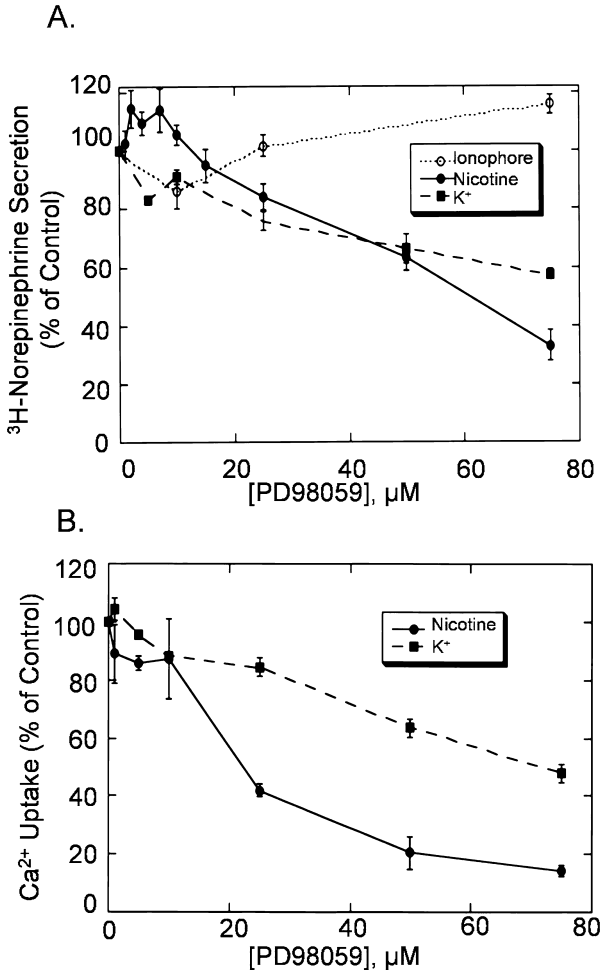

Figure 3. PD98059 blocks the release of norepinephrine from chromaffin cells in a manner independent of ERK1/2 inhibition. (A) Cells were stimulated with either 10 μM nicotine, 59 mM KCL, or 10 μM ionomycin for 10 min in the presence of PD98059 ($\leq$75 μM). 3H-Norepinephrine release is expressed as a percent of the release in the absence of the drug. Data are means $\pm$ the standard error of the mean (sem, bars) from five (nicotine), four (K^+), and three (ionomycin) experiments, each conducted in triplicate. (B) $^{45}Ca^{2+}$ uptake measured after 10 min stimulations with either 10 μM nicotine or 59 mM KCl as a function of PD98059 concentration. The data are presented as a percent of the uptake in the absence of the drug. The rate of uptake in the absence of PD98059 varied between 1.5 and 2 fmol of ^{45}Ca/cell. Data are means $\pm$ sem from three experiments for each secretagogue, each conducted in triplicate.

The concentrations of these inhibitors that activate AMPK are similar to those that we observed to inhibit calcium entry. Thus, it possible that these two off-target effects are mechanistically connected. Given our data reported here and the recent report in which doses of both PD98059 and U0126 commonly used to inhibit MEK1/2 reduced the rate of mitochondrial respiration,[14] results from experiments using these drugs should be interpreted cautiously.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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