

Response to Comment on "PDK1 Nucleates T Cell Receptor–Induced Signaling Complex for NF- κ B Activation"

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In their comment, Gruber *et al.* report constitutive phosphorylation of protein kinase C θ at threonine residue 538. However, they fail to note that, consistent with our results, a number of other groups have previously reported inducible phosphorylation of Thr-538 in T cells. Although the physiological relevance of this discrepancy is unclear, substantial differences in experimental conditions and reagents may account for the conflicting observations.

Gruber *et al.* (1) report that they observe constitutive phosphorylation of protein kinase C θ (PKC θ) at threonine residue 538 (Thr-538). However, our report (2) is not the first to demonstrate inducible phosphorylation of PKC θ on Thr-538 in T cells (3–5). Gruber *et al.* have also reported constitutive phosphorylation in previous publications (6, 7). Clearly, investigators are divided on whether Thr-538 phosphorylation is inducible or constitutive, with the caveat that, to date, reports of constitutive phosphorylation have been based mostly on overexpression experiments. Therefore, although the comment by Gruber *et al.* finally showing an example in which endogenous PKC θ appears to be constitutively phosphorylated at Thr-538 in T cells is interesting, the usefulness of this data as it applies to our findings is limited because their experiments are not technically comparable to our own.

Gruber *et al.* (1) describe an experiment in which they transiently transfect Jurkat T cells with PKC θ -expressing constructs and examine the status of phosphorylation of the expressed protein [figure 2 in (1)]. However, we fail to see how such an experiment might work. It is well known that transient transfection of Jurkat cells is an inefficient process and that only a fraction of cells can be transfected. Therefore, it seems that the signal from the endogenous PKC θ should overwhelm that of transfected PKC θ unless, on a per cell basis, PKC θ was grossly overexpressed. Likewise, if transfection is to be used, it is necessary to assess activation in transfected cells rather than observing total extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation as was done in their experiment. It is also important to point out that in the experiment shown in figure 1 in (1), where Gruber *et al.* claim to observe basal phosphorylation from primary CD3⁺ mouse cells, their experimental protocol differs substantially from ours. T cell

receptor (TCR) stimulation in our experiments was performed using soluble cross-linked antibodies rather than plate- or bead-bound antibody, a difference that may well affect TCR signaling through PKC θ (8). Our analysis in primary T cells was performed using CD4⁺ T cells, whereas Gruber *et al.* used whole CD3⁺ mouse splenocytes obtained by negative selection. In addition, it is unclear whether they ensured that the isolated T cells were in a quiescent state by controlling the quality and quantity of serum in the medium. We argue that abruptly altering culture conditions from 10% to 0.5% serum is not an appropriate method of addressing this issue.

Although the experiments provided by Gruber *et al.* are difficult to compare directly with ours, we are perplexed by their observations regarding Thr-538 phosphorylation in unstimulated cells. In

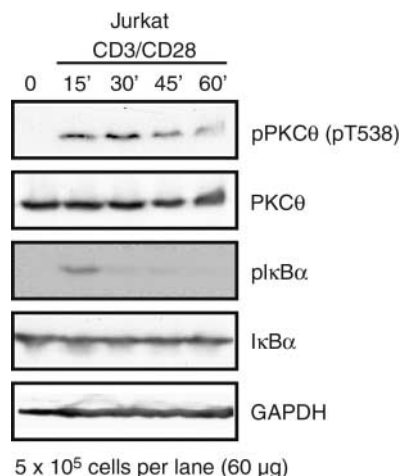


Fig. 1. Jurkat cells were stimulated as described previously and after centrifugation were lysed directly with radioimmunoprecipitation assay (RIPA) buffer (containing protease and phosphatase inhibitors) for 15 min on ice. After addition of SDS-loading buffer, equal amounts of the samples were fractionated on SDS–polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene fluoride (PVDF) membranes. Immunoblotting of the membrane was carried out using standard protocols (BD Pharmingen).

the experiments that we published (2), PKC θ was immunoprecipitated before immunoblotting, raising the somewhat unlikely possibility that there might have been selective loss of phosphate from the PKC θ from unstimulated cells during the time required for immunoprecipitation. We have therefore repeated the analysis with straightforward immunoblotting of extracts from unstimulated and stimulated Jurkat cells. As shown in Fig. 1, extracts from unstimulated cells do not show phosphorylation of PKC θ on Thr-538, whereas stimulation leads to a striking increase in phosphorylation. Furthermore, we have consistently obtained similar results using pT538 antibodies from two different vendors and in multiple Jurkat cell stocks. We are currently in the process of generating mouse knock-ins of phosphorylation-site mutants of PKC θ —experiments that we believe will unequivocally establish the importance of phosphorylation in regulation of PKC θ .

So, what might be the basis of the difference between our results and those reported by Gruber *et al.*? One factor to which we pay particularly close attention is the basal state of activation of cells. It has been our experience that Jurkat cells in particular can be easily stimulated by different batches of serum. We therefore characterize their state of activation by following the degree of basal nuclear factor κ B (NF- κ B) activity, as measured by electrophoretic mobility shift assay or luciferase reporter assays. Demonstrating inducible ERK phosphorylation does not necessarily ensure that Jurkat cells are not abnormally activated with regard to PKC θ -dependent signaling pathways. It might be helpful for the authors to examine the state of activation in their unstimulated cells (e.g., NF- κ B activity, PI-3 kinase activity) before stimulating the cells with antibodies to CD3 and CD28. Nevertheless, despite the difficulties in comparing our data to that generated using disparate experimental approaches, there is ample evidence, both here and elsewhere in the literature, to suggest that the sequence and importance of individual PKC θ phosphorylation events does require further clarification. Therefore, in our own research, we continue to investigate this issue by generating knock-in mouse strains, as well as through accurate repetition of the experiments of Gruber *et al.* and others to determine both the origin and the importance of the discrepancies discussed herein.

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