REPLY TO MASIN ET AL:

# To be or not to be a phospholipase A

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In their letter, Masin et al. (1) affirm that a "varying extent of contamination" of adenylate cyclase toxin (ACT) and ACT-S606A purified toxin preparations, by the phospholipase A (PLA) enzyme from the outer membrane (OMPLA) of the producing *Escherichia coli* cells, could easily account for our finding that ACT has intrinsic PLA activity (2). *E. coli* OMPLA is an integral membrane protein of very low abundance (~200–500 copies per cell) (3), whose extraction from the bacterial membrane is particularly difficult, requiring harsh detergents (SDS) for its extraction-solubilization (3–6). In its native environment, OMPLA is inactive (5) and its activation requires, in vitro, addition of detergents (TX-100), polymyxin B, melittin, and in vivo, heat shock or colicin export (2). OMPLA has lysophospholipase activity (3–6), and has two calcium binding sites with micromolar affinity (7). What Masin et al. (1) argue involves that the "putative contaminant" is not randomly present in our preparations, but rather that it would have to be, precisely, "more" present in ACT, and "less" present in S606A, and absent (?) in D1079A preparations, to fit our data. The PLA activity we characterized in our purified ACT preparations has no lysophospholipase activity and its activity strictly depends on calcium at the millimolar range.

Recently, another toxin of the RTX family, *Vibrio cholerae* MARTX, with sequence homology with ACT, has been reported that has $PLA_1$ activity (8). A putative contamination of our toxin by OMPLA seems, therefore, unconvincing, and even if it happened it would be an "inactive contaminant." Regarding the supposition of loss of RTX domain function of ACT-D1079A by protein degradation, mentioned in the Masin et al. (1) letter, our data (CD, binding to liposomes and to macrophages, and calcium response) do not support it. In relation to the methyl arachidonyl fluorophosphonate (MAFP)-treated ACT data in cells, we show that control ACT (no MAFP) treated identically (dialysis) retains its PLA activity in cells, and the inhibitory effect of MAFP on the ACT-PLA activity was also proved in the PLA assays in vitro, in which treatment of the samples did not include the dialysis step. Furthermore, the MAFP-induced inhibition is not the single proof to conclude that ACT has PLA activity and that this activity is involved in translocation; the data with the PLA-inactive ACT-S606A mutant lead to the same conclusions. Regarding previous data by Osicka et al. (9) showing that insertion of a tripeptide at position 607 of ACT sequence, adjacent to Ser-606, has no effect on AC delivery, Masin et al. (1) failed to mention that insertion of longer peptides (nona- and undecapeptides) does reduce AC translocation.


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