Abstract

The M_3 muscarinic acetylcholine receptor (mAChR), a typical G protein-coupled receptor, expressed in HEK-293 cells stimulates phospholipase D in a pertussis toxin (PTX)-insensitive manner and the PLD response to the M_3 mAChR requires ARF and Rho GTPases and the Rho-activated Rho-kinase. However, Rho-kinase did not phosphorylate PLD enzymes directly, suggesting additional components mediate the PLD regulation by Rho/Rho-kinase. In this thesis, **first,** by transient expression of α-subunits of the PTX-resistant G proteins, G_q, G_{12} and G_{13} (wild-type, constitutively active and dominant-negative mutants), evidence is provided that the M_3 mAChR specifically couples to PLC *via* $G\alpha_q$ and to PLD *via* the G_{12}-type G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, which are apparently both required for full PLD stimulation. These data were confirmed by expression (transient or by infection with recombinant adenoviruses) of RGS4 or Lsc-RGS, which act as specific GTPase-activating proteins for $G\alpha_q$- and $G\alpha_{12}$-type G proteins, respectively. **Second,** the expression of catalytically inactive PLD1 reduced specifically PLD stimulation by M_3 mAChR, whereas its counterpart of PLD2 inhibited only PLD stimulation by PKC and EGF, suggesting that the G protein-coupled receptors signals primarily to PLD1, whereas the tyrosine kinase receptors signal *via* PKC to PLD2. **Third,** expression of wild-type and constitutively active LIM-kinase, a Rho-kinase effector, potentiated PLD stimulation by the M_3 mAChR, whereas kinase-deficient LIM-kinase had the opposite effect. Purified recombinant LIM-kinase stimulated PLD activity in cell membranes, similar as but not additive with activated RhoA or Rho-kinase. In addition, PLD stimulation by constitutively active LIM-kinase, but not by wild-type LIM-kinase, was resistant to inactivation of Rho and Rho-kinase, whereas PLD stimulation by constitutively active Rho-kinase was fully abolished by kinase-deficient LIM-kinase. Furthermore, LIM-kinase neither directly interacts with nor phosphorylates PLD enzymes, suggesting that some undefined component is involved in PLD regulation by Rho-kinase/LIM-kinase. We found that expression of wild-type cofilin, an actin depolymerization factor (LIM-kinase substrate) potentiated PLD stimulation by the M_3 mAChR, whereas the nonphosphorylatable cofilin mutant, S3A cofilin, reduced the receptor response. And this PLD stimulation by cofilin was suppressed by inactivation of Rho or Rho-kinase. *In vitro*, cofilin protein, but not its S3A mutant, specifically interacts with PLD1 and strongly increases the activity of PLD1 upon phosphorylation by LIM-kinase. In addition, expression of wild-type cofilin, but not S3A cofilin, specifically redistributed PLD1 to the plasma membrane. **Taken together,** we demonstrated that stimulation of PLD by G protein-coupled receptors, known to involve ARF and Rho GTPases and the Rho-activated Rho-kinase, is mediated specifically by heterotrimeric G proteins of the G_{12}-subtype ($G\alpha_{12}$ and $G\alpha_{13}$), the Rho-kinase effector, LIM-kinase, and the LIM-kinase substrate, cofilin, which apparently in its phosphorylated form interacts with and stimulates PLD1 activity.

Key words: phospholipase D**,** G protein-coupled receptor, G proteins
M_3 muscarinic acetylcholine receptor, LIM-kinase, Cofilin,