Dissociation of Functional Roles of Dynamin in Receptor-mediated Endocytosis and Mitogenic Signal Transduction*

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Dynamin plays a critical role in the membrane fission mechanism that mediates regulated endocytosis of many G protein-coupled receptors. In addition, dynamin is required for ligand-induced activation of mitogen-activated protein kinase by certain receptors, raising a general question about the role of dynamin in mitogenic signal transduction. Here we report that endocytosis of $\mu$ and $\delta$ opioid receptors is not required for efficient ligand-induced activation of mitogen-activated protein kinase. Nevertheless, mitogenic signaling mediated by these receptors is specifically dynamin-dependent. Thus a functional role of dynamin in mitogenic signaling can be dissociated from its role in receptor-mediated endocytosis, suggesting a previously unidentified and distinct role of dynamin in signal transduction by certain G protein-coupled receptors.

Dynamin is a cytoplasmic GTPase that plays an essential role in receptor-mediated endocytosis via clathrin-coated pits and caveolae (1, 2). Dynamin is required for constitutive endocytosis of many membrane proteins as well as ligand-induced endocytosis of numerous receptors that mediate signal transduction, including receptor tyrosine kinases such as the epidermal growth factor receptor (3) and G protein-coupled receptors such as adrenergic (4) and opioid receptors (5, 6). The rapid removal of ligand-activated receptors from the cell surface mediated by this mechanism plays a well-established role in functional desensitization of receptor-mediated signal transduction (7, 8). Recent studies indicate that ligand-induced activation of mitogen-activated protein (MAP)1 kinase both by receptor tyrosine kinases, such as the epidermal growth factor receptor (3), and G protein-coupled receptors, such as the $\beta_2$ adrenergic receptor (9), is also specifically inhibited by dominant-negative mutant dynamin. These observations have been interpreted to indicate that endocytosis of a wide variety of signaling receptors is a general requirement for receptor-mediated signal transduction via mitogen kinase cascades (3, 9).

Recent studies have demonstrated that the $\alpha_{2A}$ adrenergic receptor, $\kappa$ opioid receptor and m3 muscarinic receptor can signal to the MAP kinase cascade in the absence of detectable receptor endocytosis (10–12). However, both $\alpha_{2A}$ adrenergic and $\kappa$ opioid receptor signaling to MAPK was not blocked by dominant-negative mutant dynamin (10, 11), suggesting these receptors can signal to MAPK through a dynamin-independent mechanism. In addition, although the effect of dominant-negative dynamin on the ability of the m3 muscarinic receptor to signal to MAPK was not examined, the m3 muscarinic receptor is coupled to $G_{\alpha11}$, which presumably activates MAPK via protein kinase C phosphorylation (13).

Opioid receptors couple via $G_i$ to a variety of downstream effectors, including adenylyl cyclase (14, 15) and MAP kinase (16–18). Whereas these receptors are activated both by endogenously produced peptide ligands and by clinically important alkaloid drugs such as morphine and heroin, individual agonists differ significantly in their ability to induce dynamin-dependent endocytosis of the $\mu$ opioid receptor. Peptide agonists induce rapid endocytosis of $\mu$ opioid receptors via clathrin-coated pits (19, 20). In marked contrast, opioid receptors remain in the plasma membrane and are highly resistant to endocytosis following activation by alkaloid agonist drugs such as morphine (19, 21). This pharmacological dissociation between receptor activation and endocytosis can be observed in native neurons (21, 22) and is associated with differences in the regulation of opioid receptor-mediated signal transduction (5, 23). A requirement of receptor endocytosis for mitogenic signal transduction therefore suggests that opiate drugs such as morphine may be profoundly deficient in mitogenic signaling activity. However, morphine is capable of causing significant activation of MAP kinase via pertussis toxin-sensitive G proteins (16, 24). Furthermore, signaling via MAP kinase contributes to some mechanisms of opioid receptor desensitization, suggesting a role of mitogenic signaling in the development of physiological tolerance to opiate drugs (24). Moreover, recent studies suggest an important role of MAP kinase signaling in the pathophysiology of physiological dependence and withdrawal following prolonged administration of morphine (18). Thus the physiological actions of addictive opiates suggest a reappraisal of the relationship between receptor endocytosis and mitogenic signal transduction.

We have addressed this issue using a well established model cell system in which both $\mu$ and $\delta$ opioid receptors are regulated by dynamin-dependent endocytosis (5, 6), and in which the agonist selectivity of opioid receptor endocytosis closely parallels that observed in native neurons (21, 22). Our studies indicate that endocytosis of $\mu$ and $\delta$ opioid receptors is not required for efficient receptor-mediated activation of MAP kinase. Nevertheless, opioid receptor-mediated activation of MAP kinase measured in the same cells is strongly and specifically dynamin-dependent. These observations suggest a role of dynamin in mitogenic signal transduction mediated by certain G protein-coupled receptors that is distinct from its role in mediating endocytosis of the receptor itself.

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† The abbreviations used are: MAP, mitogen-activated protein; MAPK, MAP kinase; HEK, human embryonic kidney.
The Role of Dynamin in Endocytosis and MAPK Signaling

**EXPERIMENTAL PROCEDURES**

**Immunocytochemical Staining and Fluorescence Microscopy—**Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown on coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (University of California, San Francisco, cell culture facility). Stably transfected cells expressing μ opioid receptors were generated by calcium phosphate precipitation and selected with 100 μg/ml geneticin (Life Technologies, Inc.) For staining, cells were incubated for 30 min in medium containing 3.5 μg/ml M1 anti-FLAG antibody (Kodak IBI), then treated with 5 μM agonist for 30 min, and fixed using 4% formaldehyde in phosphate-buffered saline. Cells were permeabilized in 0.1% Triton X-100 (Sigma) in 3% dry milk in Tris-buffered saline + 1 mM CaCl₂, then incubated with Cy3-conjugated donkey anti-mouse secondary antibody for 30 min (1:500, Jackson Immunoresearch Laboratories, Inc.). Conventional fluorescence microscopy was performed using a Nikon 60X NA1.4 objective.

**Detection of MAP Kinase and Phospho-MAP Kinase by Immunoblotting—**Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum then starved without serum for 18 h. Cells were then cultured for 2 h in fresh serum free medium, then exposed to new serum free medium containing agonist for 5 min. Cells were washed twice with phosphate-buffered saline and lysed in SDS sample buffer. Samples were passed through a 22-gauge needle to reduce sample viscosity, heated to 100 °C for 5 min, cooled on ice, then separated by SDS-polyacrylamide gel electrophoreses, transferred to nitrocellulose, and detected by immunoblotting according to the manufacturer’s instructions (NEB, Inc, Beverly MA).

**RESULTS AND DISCUSSION**

Ligand-dependent activation of MAP kinase was assayed in stably transfected HEK293 cells expressing murine μ opioid receptors using an antibody that specifically recognizes the activated form of p42/44 MAP kinase (Erk1 and Erk2). We compared the effects of opioid peptide (D-Ala², N-methyl-Phe⁴, Gly-ol⁴)enkephalin, a hydrolysis-resistant analog of enkephalin) to those of the alkaloid analgesic drugs morphine and etorphine. Both of these drugs activate opioid receptors to signal via heterotrimeric G proteins, but differ dramatically in their effects on dynamin-dependent endocytosis of receptors (5, 6, 19). Morphine caused no detectable endocytosis of receptors, whereas etorphine promoted dynamin-dependent endocytosis of receptors to a similar extent as opioid peptide (Fig. 1A). Surprisingly, despite their dramatically different effects on receptor endocytosis, each of these agonists strongly induced receptor-mediated activation of MAP kinase (Fig. 1B). Furthermore, MAP kinase activation induced by all these agonists occurred with similar kinetics (not shown), causing peak activation at 5 min after addition to the culture medium. Quantitation of MAP kinase activation at this time point indicated that all of these ligands (when examined at saturating concentrations to avoid confounding effects of differences in agonist potency) (15, 25) were similarly efficacious for inducing receptor-mediated activation of MAP kinase (Fig. 1C).

We next asked whether the ability of morphine to activate MAP kinase without inducing endocytosis of receptors was unique to this agonist, or whether this property may be more general to other analgesic drugs. Furthermore, as many analgesic drugs activate more than one type of opioid receptor when administered at clinically relevant concentrations, we also asked whether other opioid receptors were capable of mediating endocytosis-independent activation of MAP kinase. To accomplish this, we examined a chimeric mutant δκ opioid receptor that is completely defective in endocytosis, even when activated by saturating concentrations of etorphine (6). Despite its failure to endocytose upon activation by agonist, this mutant opioid receptor mediated etorphine-induced activation of MAP kinase to an equal or greater extent than did the wild-type δ opioid receptor (Fig. 2). Thus the ability of opiate drugs to induce endocytosis-independent activation of MAP kinase is not unique to morphine or the μ opioid receptor.

As an alternative test of a potential role of opioid receptor endocytosis in mitogenic signaling, we examined whether experimentally inducing dynamin-dependent endocytosis of mor-
phine-activated opioid receptors was sufficient to enhance drug-induced activation of MAP kinase. To accomplish this, we examined receptor-mediated MAP kinase activation in cells overexpressing β-arrestin at high levels, in which morphine promotes rapid, dynamin-dependent endocytosis of opioid receptors (5). Despite the profoundly different effects of morphine on opioid receptor endocytosis observed in this variant cell line compared with cells expressing receptors at similar level but not overexpressing β-arrestin, no enhancement of morphine-induced activation of MAP kinase was observed (Fig. 3, compare lanes 4 and 7). Furthermore the amount of MAP kinase activation induced by morphine was closely similar to that induced by opioid peptide in both cell lines (Fig. 3, compare lanes 3 and 4 with lanes 6 and 7), even though morphine-induced endocytosis of opioid receptors is selectively enhanced by >40-fold in the cells overexpressing arrestin (5). Taken together, these experiments indicate that dynamin-dependent endocytosis of opioid receptors is neither necessary nor sufficient for receptor-mediated activation of MAP kinase. Despite the ability of opiate drugs to strongly induce endocytosis-independent activation of MAP kinase, previous studies support an essential role of dynamin-dependent endocytosis of other G protein-coupled receptors in MAP kinase signaling. Furthermore, recent studies indicate that MAP kinase activation induced by opioid peptides is also strongly dynamin-dependent (26). Thus we examined whether opioid receptor-mediated activation of MAP kinase induced by opiate drugs also required functional dynamin. To accomplish this, opioid-induced activation of MAP kinase was assayed in cells overexpressing K44E mutant dynamin I, which specifically inhibits endocytosis via clathrin-coated pits in a dominant-negative manner (1, 2). Surprisingly, these experiments clearly indicated that receptor-mediated activation of MAP kinase induced by opiate drugs was also strongly and specifically dynamin-dependent. Receptor-mediated activation of MAP kinase induced by both etorphine and morphine was completely blocked by K44E mutant dynamin (Fig. 4) even though morphine failed to induce any detectable endocytosis of receptors over the time course of MAP kinase activation. Overexpressing wild-type dynamin I at similar levels did not block opioid receptor-mediated activation of MAP kinase. To study the role of dynamin in endocytosis and MAPK signaling in these cells, we examined receptor-mediated MAP kinase activation in cells expressing opioid receptors and either wild-type or K44E mutant dynamin (1, 2) or wild-type dynamin, and subsequently assayed for agonist-induced activation of MAP kinase. The efficiency of dynamin transfection and inhibition of ligand-induced endocytosis (both >75%) was verified by fluorescence microscopy in control experiments (not shown). K44E mutant dynamin completely inhibited isoproterenol-induced activation of MAP kinase mediated by the β2-adrenergic receptor, as reported previously by others. K44E mutant dynamin also blocked etorphine- and morphine-stimulated activation of MAP kinase mediated by the μ opioid receptor. In contrast, overexpression of wild-type dynamin at similar levels did not block receptor-mediated activation of MAP kinase. Despite the ability of opiate drugs to strongly induce endocytosis-independent activation of MAP kinase, previous studies support an essential role of dynamin-dependent endocytosis of other G protein-coupled receptors in MAP kinase signaling. Furthermore, recent studies indicate that MAP kinase activation induced by opioid peptides is also strongly dynamin-dependent (26). Thus we examined whether opioid receptor-mediated activation of MAP kinase induced by opiate drugs also required functional dynamin. To accomplish this, opioid-induced activation of MAP kinase was assayed in cells overexpressing K44E mutant dynamin I, which specifically inhibits endocytosis via clathrin-coated pits in a dominant-negative manner (1, 2). Surprisingly, these experiments clearly indicated that receptor-mediated activation of MAP kinase induced by opiate drugs was also strongly and specifically dynamin-dependent. Receptor-mediated activation of MAP kinase induced by both etorphine and morphine was completely blocked by K44E mutant dynamin (Fig. 4) even though morphine failed to induce any detectable endocytosis of receptors over the time course of MAP kinase activation. Overexpressing wild-type dynamin I at similar levels did not block opioid receptor-mediated activation of MAP kinase.
ated activation of MAP kinase by either agonist (Fig. 4), confirming the biochemical specificity of the inhibition observed.

We conclude that endocytosis is not required for efficient ligand-induced activation of MAP kinase by μ and δ opioid receptors, even though receptor-mediated signaling via this effector cascade is specifically dynamin-dependent. These studies establish the first example of MAP kinase activation by a G protein-coupled receptor that is dynamin-dependent but does not require endocytosis of the receptor protein. Thus these studies suggest an additional function of dynamin in mitogenic signal transduction that is distinct from its essential role in mediating endocytosis of the receptor itself.

Further studies will be required to elucidate the precise role of dynamin in mitogenic signal transduction. One possibility is that dynamin-dependent endocytosis of another protein, distinct from the opioid receptor itself, is required for receptor-mediated activation of MAP kinase. Another possibility is that dynamin may mediate a distinct biochemical function in signal transduction in addition to its essential role in endocytic membrane fission (27–29). In support of this latter hypothesis, we note that dynamin is a regulated GTPase that is capable of interacting with a wide variety of cytoplasmic and membrane-associated proteins. Although some of these interacting proteins are associated specifically with clathrin-coated pits, others (such as amphiphysin, Grb2, phospholipase Cγ, phosphatidylinositol 3-kinase, and Src) are thought to mediate distinct or additional functions independent of, or in addition to, the membrane fission machinery itself (for review see Ref. 30).

Moreover, a significant pool of cellular dynamin isoforms is associated with microdomains of the plasma membrane that are not engaged in endocytosis (2). Finally, although the traditionally accepted role of dynamin is as a mechanochemical GTPase that itself mediates endocytic membrane fission (31, 32), recent studies suggest that dynamin can function as a GTP-dependent molecular switch to regulate the membrane fission machinery in a manner analogous to the regulation of a downstream effector by a “classical” signaling GTPase (33).

Whereas previous studies provide strong evidence for significant heterogeneity in mechanisms of mitogenic signal transduction by individual G protein-coupled receptors in various cell types, the present results provide the first direct evidence for distinct functional roles of dynamin in G protein-coupled receptor-mediated endocytosis and signal transduction. These observations are specifically relevant to the physiological actions of opiate drugs such as morphine on the μ opioid receptor. However, it is clear from our studies that these observations are not unique to morphine nor to the μ opioid receptor. Moreover, as opioid receptors activate MAP kinase by a molecular mechanism similar to that of other G coupled receptors (and distinct from an alternative protein kinase C-mediated mechanism activated by Gα-coupled receptors) (13), we anticipate that this dissociation between the signaling and endocytic activities of dynamin may be of general importance to certain other G protein-coupled receptors that activate MAP kinase in a dynamin-dependent manner.

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REFERENCES

1. Herskovits, J. S., Burgess, C. C., Ohar, R. A., and Vallee, R. B. (1993) J. Cell Biol. 122, 565–578
2. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
3. Viets, A. V., Lamaze, C., and Schmid, S. L. (1996) Science 274, 2086–2089
4. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) J. Biol. Chem. 272, 27055–27064
5. Whistler, J. L., and von Zastrow, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9914–9919
6. Chu, P., Murray, S., Lissin, D., and von Zastrow, M. (1997) J. Biol. Chem. 272, 27124–27130
7. Ferguson, S. S., and Caron, M. G. (1998) Semin. Cell Dev. Biol. 9, 119–127
8. Leffkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) Adv. Pharmacol. 42, 416–420
9. Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G., and Leffkowitz, R. J. (1998) J. Biol. Chem. 273, 685–688
10. DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) J. Biol. Chem. 274, 11253–11259
11. Li, J., Luo, L., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L. (1999) J. Biol. Chem. 274, 12087–12094
12. Budd, D. C., Rae, A., and Tobin, A. B. (1999) J. Biol. Chem. 274, 12355–12360
13. Gutkind, J. S. (1998) J. Biol. Chem. 273, 1839–1842
14. Evans, C. J. (1993) in Biological Basis of Substance Abuse (Korenman, S. G., and Barchas, J. D., eds), pp. 31–48, Oxford University Press, New York
15. Raynor, K., Kong, H., Chen, Y., Yasuda, K., Yu, L., Bell, G. I., and Reisine, T. (1994) Mol. Pharmacol. 45, 330–334
16. Burt, R. P., Chappel, C. R., and Marshall, I. (1996) Br. J. Pharmacol. 117, 224–230
17. Li, L. Y., and Chang, K. J. (1996) Mol. Pharmacol. 50, 599–602
18. Schulz, S., and Hall, V. H. (1998) Eur. J. Neurosci. 10, 1196–1201
19. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) J. Biol. Chem. 271, 19021–19024
20. Trupin, N., Keith, D. E., Cvejic, S., Evans, C. J., and Defi, L. A. (1996) J. Biol. Chem. 271, 29279–29285
21. Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Monteillet-Agius, G., Stewart, P. L., Evans, C. J., and von Zastrow, M. (1999) Mol. Pharmacol. 55, 377–384
22. Sternini, C., Sspan, M., Anton, B., Keith, D. J., Bunnell, N. W., ven, Z. M., Evans, C., and Brecha, N. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9241–9246
23. Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. H., Laporte, S. A., Law, P. Y., and Caron, M. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7157–7162
24. Polakiewicz, R. D., Schiefer, S. M., Donner, L. F., Kanara, V., and Comb, M. J. (1998) J. Biol. Chem. 273, 12492–12496
25. Raynor, K., Kong, H., Law, S., Heerding, J., Tallent, M., Livingston, F., Hines, J., and Reisine, T. (1996) Natl. Inst. Drug Abuse Res. Monogr. 161, 83–103
26. Ignatova, E. G., Belcheva, M. M., Bohn, L. M., Neuman, M. C., and Coscia, C. J. (1999) J. Neurosci. 19, 56–63
27. Schmid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
28. Warnock, D. E., and Schmid, S. L. (1996) Biosci. Rep. 18, 885–893
29. Hinshaw, J. E., and Schmid, S. L. (1996) Nature 374, 190–192
30. Schmid, S. L., McNiven, M. A., and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512
31. Stertz, S. M., and Hinshaw, J. E. (1998) Cell 93, 1021–1029
32. Takei, K., Hauke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998) Cell 94, 131–141
33. Sever, S., Muhlig, A. B., and Schmid, S. L. (1999) Nature 395, 481–486