ERKI/II Regulation by the Muscarinic Acetylcholine Receptors in Neurons

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Muscarinic acetylcholine receptors (mAChRs) are known to be involved in learning and memory, but the molecular basis of their involvement is not well understood. The availability of new and specific biochemical tools has revealed a crucial role for the mitogen-activated protein kinase (MAPK) family in learning and memory. Here, we examine the link between mAChRs and MAPK in neurons. Using the MAPK kinase (MEK)-specific inhibitor PD98059, we first demonstrate a necessary role for active ERKI/II in long-term potentiation in vivo. Using phospho-specific antibodies that recognize the activated form of ERKI/II, we find that the level of ERKI/II activation in brain is regulated by mAChRs. Carbachol, a muscarinic agonist, induces prolonged activation of ERKI/II, without effect on the related kinase SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal protein kinase) in primary cortical cultures. ERKI/II activation is Src-dependent and partially phosphoinositide-3 kinase- and Ca2+-dependent but is PKC-independent. M1–M4 mAChR subtypes expressed in COS-7 cells can all induce ERKI/II activation using a signal transduction pathway similar to that operating in neurons. The nature of the signal transduction pathway suggests that ERKI/II may serve as a convergence site for mAChR activation and other neurotransmitter receptors.

Key words: mAChR; extracellular regulated kinase; MAPK; neurons; COS-7; LTP; signal transduction

Cholinergic transmission at the muscarinic acetylcholine receptor (mAChR) has been implicated in learning and memory in humans and other mammals (Blokland, 1995). The cholinergic innervation of the cerebral cortex and the hippocampus originates primarily from the cholinergic basal nuclear complex (Mesulam, 1996). Lesions of these basal forebrain neurons have been reported to result in impairment in memory, learning, and attention, whereas cholinergic agonists facilitate learning and memory (Jerusalinsky et al., 1997).

The mAChR family consists of five heterogeneous mAChR subtypes, differentially expressed in the brain. These receptors transduce their signal by coupling to G-proteins (Wess, 1993). In neurons, activation of mAChRs can induce elevation of intracellular Ca2+ and stimulate kinase activation, as well as increasing phosphoinositide turnover (Felder, 1995). It has been shown recently that, in different cell lines, expression of mAChRs can induce proliferation by activating the extracellular signal-regulated kinase (ERK) pathway (Gutkind, 1998). Multiple signal transduction pathways may mediate ERK activation by mAChRs, and there are conflicting results, probably attributed to the different cell lines used (Sugden and Clerk, 1997; Gutkind, 1998). ERK activation is both necessary for and correlated with several forms of synaptic plasticity (for review, see Orban et al., 1999), including long-term potentiation (LTP), the major cellular model of learning and memory (Bliss and Collingridge, 1993), in a rat hippocampal slice preparation (English and Sweatt, 1997). LTP in the cortex and the hippocampus is modulated by mAChRs (Jerusalinsky et al., 1997), and we have found recently that atropine attenuates cortical LTP in vivo (Jones et al., 1999).

To examine the extent to which mAChRs exert their effect on the mature brain via the modulation of ERKI/II activity, we have investigated the activation of ERKI/II by mAChRs in neuronal tissue. We have used different levels of analysis, ranging from the intact brain to primary cortical cell cultures, and a model system consisting of COS-7 cells expressing the different mAChR subtypes, to gain insight into the signal transduction linkages involved in ERKI/II activation and its physiological significance. We report here that ERKI/II activation is necessary for the expression of LTP in vivo in the dentate gyrus (DG) of the hippocampus. ERKI/II activity is modulated by mAChRs in the neocortex and hippocampus in vivo, in hippocampal slices, and in primary cortical neurons. Low doses of the muscarinic agonist carbachol can induce prolonged activation of ERKI/II but not another member of the mitogen-activated protein kinase (MAPK) family, stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK), in primary cortical neurons and in COS-7 cells expressing the different mAChRs. ERKI/II activation is independent of protein kinase C (PKC) but is blocked by inhibitors of the Src protein tyrosine kinase and is attenuated by phosphoinositide-3 kinase (PI3K) inhibitors and Ca2+ chelators. The M1–M4 mAChR subtypes can all induce ERKI/II activation when expressed in COS-7 cells and share a similar signaling pathway dependency with the neurons. Our results demonstrating that different signal transduction cascades involved in ERKI/II activation by different neurotransmitters suggest that fast (e.g., glutamergic) and modulatory (e.g., cholinergic) neurotransmission, both necessary for normal learning and memory, may converge on ERKI/II in a given neuron.
**Western blot analysis.** Aliquots in SDS–sample buffer were subjected to SDS-PAGE (Laemmli, 1970; Schagger and von Jagow, 1987) and Western blot analysis (Burnette, 1981). After the run, the blots were blocked with 1% BSA or 5% dried milk for 1 hr at room temperature. The blots were reacted either overnight in a cold room or 1 hr at room temperature with primary antibody. After three short washes, the blots were subsequently incubated for 1 hr at room temperature with HRP-linked protein A, Protein G-HRP (Zymed, San Francisco, CA), or HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Amersham). The blots were then exposed to ECL substrate and film (Amersham). The primary antibodies used were dually phosphorylated (dp) ERK1/II (1:30000 (Promega, Madison, WI); 1:5000 (New England Biolabs, Beverly, MA)), dpSAPK/JNK (1:1000; New England Biolabs), and ERK1/II (1:2000; New England Biolabs). Usually the blots were first treated with anti-dpERK1/II antibody, stripped with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7), and reprobed with anti-ERK1/II antibody.

Quantification was performed using computerized densitometry and an image analyzer (Molecular Dynamics, Sunnyvale, CA). Differences between two groups were determined using two-way Student’s t test (α level of 0.05).

**RESULTS**

**ERK1/II activation is necessary for in vivo LTP and is modulated by intrinsic muscarinic acetylcholine receptors in the cortex and the hippocampus**

ERK1/II activity has been found to be both necessary for and correlated with several forms of synaptic plasticity and learning (Orban, 1999). These include the most intensively studied cellular model of learning and memory, LTP, in a brain slice preparation (English and Sweatt, 1997). The slice preparation is different from the intact brain in two major ways: first, it does not include an intact modulatory input from distal brain areas, and second, there is mechanical damage to the cut tissue. For these reasons, we examined the role of ERK1/II in LTP in the DG of intact, anesthetized animals. Because no specific ERK1/II inhibitors are available, we examined the effect of PD98059, an inhibitor of ERK1/II kinase [MAP kinase/ERK kinase (MEK)], on the magnitude and duration of LTP in the DG. MEK is the kinase directly upstream of ERK1/II responsible for the dual phosphorylation of ERK1/II on tyrosine and threonine residues and hence its activation. PD98059 was microinjected into the DG of one hemisphere 30 min before the induction of LTP (Fig. 1A, arrowhead), and vehicle alone was injected into the contralateral hemisphere. Tetanic stimulation was then delivered 30 min later to the perforant path in both hemispheres (small arrowheads). Normalized data from four animals shows that the percentage change in the slope of field EPSP after tetanic stimulation was significantly (p < 0.04; paired t test) larger in the hemisphere injected with vehicle compared with the hemisphere injected with the MEK inhibitor PD98059 (Fig. 1A). This result suggests that ERK1/II plays a necessary role in the induction of LTP in the DG in vivo.

What might modulate the degree of ERK1/II activity in the intact brain? In neurons, ERK1/II can be activated by glutamate via Ca2+–dependent mechanisms (Xia et al., 1996). To assess the contribution of mAChRs to ERK1/II activation in the brain, we quantified the amount of ERK1/II phosphorylation in cortical and hippocampal tissue following procedures that either increased levels of acetylcholine (after intraperitoneal administration of phystostigmine, an acetylcholinesterase inhibitor) or decreased activity of mACHRs by intrinsic acetylcholine (after intraperitoneal administration of atropine, an mACHr antagonist). The doses of phystostigmine and atropine used were in the range reported to have effects on behavior (Beninger et al., 1989). ERK1/II activation is increased after injection of phystostigmine and reduced after injection of atropine (Fig. 1B). Together, these
results show that mAChRs are linked to ERK1/II activation in the hippocampus and cortex and that ERK1/II activation is necessary for LTP in vivo. We next sought to analyze the conditions and signal transduction pathways involved in ERK1/II activation by mAChRs in neurons.

Carbachol induces a dose-dependent activation of ERK1/II in brain slices, primary cortical neurons, and COS-7 cells expressing the different mAChR subtypes (M1–M4)

In our experiments on brain slices, we found that the basal level of ERK1/II activation varied between slices. This variation in basal expression may reflect a variety of causes, such as different amounts of damage to the tissue during slice preparation or maintenance. Irrespective of this variation, application of increasing doses of carbachol induced a dose-dependent activation of ERK1/II (Fig. 2A, top panel). A submicromolar dose of carbachol was enough to induce ERK1/II activation. A similar dose-dependent pattern of ERK1/II activation was seen using increasing doses of insulin, which acts on a receptor tyrosine kinase (RTK) (Fig. 2A, bottom panel). To analyze the molecular pathway involved in ERK1/II activation by mAChRs in a more stable system, we examined ERK1/II activation in primary cortical neurons (12–14 d in culture) and, in parallel, used a COS-7 cell line as a model system for the individual expression of each mAChR subtype. A similar pattern of expression of mAChRs is seen in cortical neurons in primary culture as in vivo (Eva et al., 1990; Andre et al., 1994). The COS-7 cell line does not endogenously express mAChRs but can be transiently transfected with each mAChR subtype to ascertain muscarinic subtype-specific effects on ERK1/II activation. Increasing doses of carbachol resulted in increasing activation of ERK1/II in primary cortical neurons (Fig. 2B). In the presence of 1 μM TTX, a similar amount of ERK1/II activation (ratio of 3.73 ± 0.16 over basal; n = 4) was detected after 1 hr incubation with 100 μM carbachol as was seen in the absence of TTX, suggesting that in primary cortical neurons ERK1/II activation is activity-independent. In COS-7 cells transiently expressing one or another of the M1–M4 subtypes, increasing doses of carbachol induced an increasing ERK1/II activation (Fig. 2C). ERK1/II activation was atropine-dependent in both preparations (see Fig. 5), and thus mAChR-dependent.

The differences in the sensitivity of ERK1/II activation may reflect differences in receptor expression levels and thus is not necessarily an indication of differences in the ability to activate the MAPK cascade. However, because M1, M2, and M4 are expressed at similar levels (data not shown), it is possible that M1 activates ERK1/II more efficiently than M2 and M4.

Carbachol induces prolonged activation of ERK1/II but not SAPK/JNK in primary cortical neurons and COS-7 cells

The time course of ERK1/II activation was found to be crucial for determining its effect on the differentiation of PC12 cells (Marshall, 1995). In novel taste learning for which functional mAChRs in the taste cortex are necessary, different time scales of activation were detected for ERK1/II and SAPK/JNK (Berman et al., 1998). We therefore analyzed the time course of ERK1/II activation by carbachol in primary cortical neurons. Carbachol induces prolonged ERK1/II activation (over 4 hr), peaking 30–60 min after carbachol administration, but does not activate SAPK/JNK (Fig. 3A).

In COS-7 cells expressing the different mAChR subtypes, ERK1/II was activated with a more rapid time course than in
primary neurons, peaking 10 min after carbachol administration (Fig. 5B). The difference in time course of ERK1/II activation between transfected COS-7 and neurons might be attributable to higher levels of expression in the COS-7 cells or to different signal transduction mechanisms involved in the activation and deactivation of ERK1/II in these two cell types. We favor the second explanation because, in HEK-293 cells, which express low levels of M3 mAChRs endogenously, the peak timing of ERK1/II activation is similar to that seen in COS-7 cells when expressing the different mAChR subtypes (M1–M4) (M. Futter, unpublished results).

**ERK1/II activation by carbachol is Src-dependent and PKC-independent**

The signal transduction pathway involved in ERK1/II activation by receptor tyrosine kinases and by G-protein-coupled receptors has been extensively investigated in cell lines (Sugden and Clerk, 1997). However, limited results are available for neuronal systems (Fukunaga and Miyamoto, 1998). We have used pharmacological inhibitors to assess the signal transduction pathways involved in the mAChR activation of ERK1/II in primary cortical neurons and in the COS-7 model system expressing different mAChR subtypes. In all of these experiments, inhibition was expressed as percentage with respect to the level of ERK1/II activation by 100 μM carbachol. BAPTA-AM and EGTA were applied for 1 hr and 10 min, respectively, before carbachol administration. The combined application of chelators of both intracellular and extracellular Ca2+ (BAPTA-AM and EGTA, respectively) only weakly attenuated ERK1/II activation by carbachol in both primary cultures and COS-7 cells expressing the different muscarinic receptors (Fig. 4A,B). The minimal dependency on Ca2+ is interesting given the ability of mAChRs to release Ca2+ from intracellular stores in variety of cell types (Felder, 1995). Moreover, the fact that stimulated NMDA receptors affect ERK1/II activation in a Ca2+-dependent manner (Fig. 4C) suggests that NMDA- and mAChR-mediated signaling within a neuron can converge on the ERK1/II protein via different molecular pathways.

We further analyzed the involvement of postulated kinases known to mediate effects downstream of G-protein activation. At least three kinases have been implicated in ERK activation in different cell lines: (1) PKC, converging at the level of Raf, (2) PI3K, and (3) Src, which both converge at the level of the ternary complex Shc–Grb2–Sos1 (Lopez-Illasaca et al., 1998). Addition of the PKC inhibitor BIM (1 μM) 15 min before carbachol administration did not affect ERK activation by carbachol in primary cortical neurons or COS-7 cells expressing the M1 mAChR (8 ± 8% in primary neurons; n = 9; and 20 ± 5% in COS-7 cells; n = 6) (Fig. 5B). Administration of 20 μM LY294002, an inhibitor of PI3K, 15 min before agonist activation, attenuated carbachol-mediated ERK activation by 68 ± 7% in primary cortical neurons (n = 8) and by 56 ± 7% in COS-7 cells expressing the M1 mAChR subtypes (n = 8) (Fig. 5B). Last, examined (n = 4). *p < 0.5; Student’s t test. C, Carbachol induces a dose-dependent activation of ERK1/II in COS-7 cells expressing the different mAChRs (M1–M4). Cells were incubated for 10 min with a given concentration of carbachol, and the degree of ERK1/II activation produced by that dose was expressed as a percentage of the maximal activation produced by 1 ng/ml epidermal growth factor (defined as 100%) (n = 4). Representative blots from cells expressing the M1 and M2 receptors are shown at the left. *p < 0.5; Student’s t test.
addition of 10 μM PP1, an inhibitor of the Src family of tyrosine kinases, 15 min before carbachol administration, attenuated ERK activation by carbachol by 91.67% in primary cortical neurons (n = 8) and by 103.62% in COS-7 cells expressing the M1 mAChR subtypes (n = 8) (Fig. 5A). The values of inhibition for COS-7 cells expressing the M1 mAChR described above were similar to values of inhibition for COS-7 cells expressing the M2 mAChR (data not shown).

The above results demonstrate the likely involvement of PI 3K and the Src family of tyrosine kinases in regulating activation of the ERK pathway by mAChRs. Several Src families are highly expressed in the brain and have been found to be obligatory for LTP in the hippocampus (Salter, 1998). In particular, Fyn, a member of the Src family, has been implicated in LTP (Grant et al., 1992). We thus analyzed ERK activation by carbachol in primary cortical cultures from Fyn knock-out mice. However, carbachol retained its ability to induce a dose-dependent ERK activation in these cultures (Fig. 5C).

**DISCUSSION**

The results presented here reveal strong biochemical connections in neurons between two classes of molecules involved in learning and memory, mAChRs and the MAPKs. There is a good deal of evidence linking mAChRs to cognitive processes, such as learning and memory (Blokland, 1995), but their role at the cellular and molecular levels in these processes is less clear. The development of new reagents for studying the MAPK signal transduction pathway (e.g., phospho-specific antibodies and selective MEK inhibitors) has led to studies suggesting specific roles for ERKII in learning and memory (Berman et al., 1998; Blum et al., 1999) and synaptic plasticity (English and Sweatt, 1997; Martin et al., 1997; Coogan et al., 1999). Here, we demonstrate that the MEK inhibitor PD98059 blocks LTP, indicating that activation of ERKII plays an obligatory role in the induction of LTP in the dentate gyrus in vivo. We also show that physostigmine increased, and atropine decreased, endogenous ERKII activity, thus establishing a physiological connection between mAChR occupancy and ERKII activity in the cortex and hippocampus. We characterize the dose–response relationship and time course of the mAChR-mediated activation of ERKII and SAPK/JNK. Finally, we characterize the signal transduction pathway involved in mAChR-mediated activation of ERKII in neurons and in COS-7 cells expressing one or another of the different mAChR subtypes. In both neurons and fibroblasts, this activation is Src-dependent but only partially dependent on PI3K and Ca\(^{2+}\). ERKII activation by carbachol is not, however, PKC-dependent. These findings demonstrate a crucial role for ERKII activation in synaptic plasticity in the intact animal and identify ERKII as a target for mAChR-mediated action in neurons. The available data suggests that, in neurons, stimuli from different receptors can converge on ERKII (Fig. 6).
area CA1 or DG of the hippocampal slice. Our experimental design, with vehicle injected into one hemisphere and PD98059 into the other, ensures that any difference in LTP is attributable to the drug. The drug at the concentration used here has been found to be highly selective (Alessi et al., 1995). This, together with the fact that ERK I/II is the only known substrate of MEK, allows us to conclude that activation of ERK I/II is necessary for the successful induction of LTP in the dentate gyrus of the intact animal (Fig. 1A). Potentiation in the hemisphere treated with the MEK inhibitor PD98059 was reduced in the initial magnitude relative to the control hemisphere and decayed to baseline over a period of ~20 min. Thus, in the hippocampus, as in the insular cortex (Jones et al., 1999), block of ERK I/II activation prevents both early and late phases of LTP, leaving only a residual period of short-term potentiation.

Pharmacological measurements in the behaving animal have established that novel stimuli can cause a release of acetylcholine in the cortical area (Acquas et al., 1996). We imitated this increase in release of acetylcholine by bath application of the mAChR agonist carbachol to hippocampal slices and to primary cortical cells in culture. Carbachol (100 μM) induces ERK I/II activation in primary cortical cultures and in COS-7 cells expressing the M1 receptor. Activation was blocked by 100 μM atropine or 10 μM PPI and attenuated by 100 μM BAPTA-AM. The blot is representative of triplicates from three different experiments. B. The PI3K inhibitor LY294002 attenuates ERK I/II activation by carbachol, but the PKC inhibitor BIM is ineffective. Carbachol (100 μM) induces ERK I/II activation in primary cortical cultures and COS-7 cells expressing the M1 receptor. The activation was blocked by the MEK inhibitor PD98059 (19 μM), attenuated by the PI3K inhibitor LY294002 (10 μM), and unaffected by the PKC inhibitor BIM (1 μM). The blot is representative of triplicates from three different experiments. C. Fyn is not necessary for ERK I/II activation by carbachol. Primary cortical cultures were prepared from Fyn knock-out mice. The cultures show dose-dependent activation of ERK I/II by carbachol.
to modulate transcription in neurons. The block of LTP induction by the MEK inhibitor PD98059 (Fig. 1A) suggests that MAPK plays a role in the early phase of LTP, which is dependent on modulation of membranal or cytosolic proteins. Another documented pathway by which MAPK is activated in neurons is via glutamate receptors, represented here by the NMDA receptor, activation of which leads to increased levels of intracellular Ca$^{2+}$.

Increased intracellular Ca$^{2+}$ may modulate the MAPK cascade via activation of a Ca$^{2+}$-dependent tyrosine kinase (PYK2) and calmodulin (CaM). The prolonged duration of MAPK activation suggests that convergence from different extracellular stimuli may take place in a time domain of minutes to hours.

In neurons and PC12 cells, ERKII can be activated via increased cytosolic Ca$^{2+}$ (Xia et al., 1996). mAChRs can increase intracellular Ca$^{2+}$ by either membrane depolarization with consequent activation of voltage-gated Ca$^{2+}$ channels or release from intracellular stores (Felder, 1995). We tested the ability of both the intracellular Ca$^{2+}$ chelator BAPTA-AM and the extracellular Ca$^{2+}$ chelator EGTA to inhibit ERKII activation by mAChR. Significantly, the Ca$^{2+}$ chelators abolished the NMDA-dependent, but not the mAChR-dependent, ERKII activation (Fig. 4), indicating that an alternative signal transduction pathway is involved in ERKII activation by mAChR.

We were interested in the role of three kinases known to be downstream of G-proteins and upstream of ERKII: PKC, PI3K, and the Src family of tyrosine kinases. Using kinase-specific inhibitors, we found that, in both cortical neurons and COS-7 cells expressing the different mAChR subtypes, ERKII activation is Src-dependent and partially PI3K- and Ca$^{2+}$-dependent but PKC-independent (Fig. 5).

What might be the signal transduction pathway involved in Ca$^{2+}$- and PKC-independent ERKII activation? In cell lines, different G-protein-coupled receptors can activate ERKII via G$^{\alpha}$ or G$^{\beta\gamma}$-proteins through the $\alpha$ and $\beta\gamma$ subunits (Crespo et al., 1994; Koch et al., 1994). Activation by G$^{\alpha}$ in different cell lines has been found to be PKC- and/or Ca$^{2+}$-dependent (Hawes et al., 1995; Della Rocca et al., 1999). The control of ERKII activation by mAChRs seems similar in neurons and COS-7 cells.

In both, there is a central role for Src kinase and an involvement of PI3K and the Src family of tyrosine kinases. Using kinase-specific inhibitors, we found that, in both cortical neurons and COS-7 cells expressing the different mAChR subtypes, ERKII activation is Src-dependent and partially PI3K- and Ca$^{2+}$-dependent but PKC-independent (Fig. 5).

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proteins similar to those used by RTKs, is dependent on G$_{b}$$gamma$-mediated tyrosine kinase activation (Fig. 6). Indeed, we have observed a general increase in tyrosine phosphorylation in brain (Rosenblum et al., 1996) hippocampal slices and in primary neurons after activation of the mAChR (K. Rosenblum and M. Futter, unpublished data).

What might be the crucial role played by ERKI/II in synaptic plasticity? We suggest that the ERKI/II can serve as a point of convergence between different signal transduction cascades in fully differentiated neurons to produce plasticity. BNDF acting on the TrkB receptor (Lu and Figurov, 1997), carbachol acting on the mAChRs (Auerbach and Segal, 1996), and glutamate acting on the NMDA receptor (Collingridge et al., 1983) can all induce LTP and strong ERKI/II activation. It would be interesting to explore the ERKI/II dependency of other two forms of LTP, i.e., those mediated by carbachol and BDNF. ERKI/II can thus serve as the biochemical integration point for the ionotropic neurotransmission represented in our model by the NMDA receptor and the modulatory transmission represented by the mAChR (Fig. 6). From the intracellular perspective, ERKI/II serves as a modulatory transmission represented by the mAChR as the biochemical integration point for the ionotropic neurotransmission alone (minutes to hours). Regarding the specific biochemical role ERKI/II plays in synaptic plasticity, the processes can take place is much longer than the one used by fast neurotransmission alone (minutes to hours). Regarding the specific biochemical role ERKI/II plays in synaptic plasticity, the results from the novel taste learning paradigm and from LTP in the CA1 region of the hippocampus suggest that ERKI/II inactivation (Atkins et al., 1998; Berman et al., 1998) produces similar results to the inhibition of protein synthesis (Frey et al., 1988; Rosenblum et al., 1993). Protein synthesis dependency provides biochemically the definition of long-term memory or potentiation: that is, a block of long-term memory and late-phase LTP, leaving short-term memory and early-phase LTP unaffected. ERKI/II is also have immediate cytosolic targets, which affect plasticity. It will be of interest to identify the downstream targets of ERKI/II in mature neurons during long- and short-term synaptic modulation (Fig. 6).

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