Activity-dependent CREB phosphorylation: Convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway

Gang-Yi Wu*, Karl Deisseroth†, and Richard W. Tsien*‡

*Department of Molecular and Cellular Physiology and †Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305

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The cAMP-responsive element binding protein (CREB), a key regulator of gene expression, is activated by phosphorylation on Ser-133. Several different protein kinases possess the capability of driving this phosphorylation, making it a point of potential convergence for multiple intracellular signaling cascades. Previous work in neurons has indicated that physiologic synaptic stimulation recruits a fast calmodulin kinase IV (CaMKIV)-dependent pathway that dominates early signaling to CREB. Here we show in hippocampal neurons that the fast, CaMK-dependent pathway can be followed by a slower pathway that depends on Ras/mitogen-activated protein kinase (MAPK), along with CaMK. This pathway was blocked by dominant-negative Ras and was specifically recruited by depolarizations that produced strong intracellular Ca^{2+} transients. When both pathways were recruited, phosphorylated CREB (pCREB) formation was overwhelmingly dominated by the CaMK pathway between 0 and 10 min, and by the MAPK pathway at 60 min, whereas the two pathways acted in concert at 30 min. The Ca^{2+} signals that produced only rapid CaMK signaling to pCREB or both rapid CaMK and slow MAPK signaling deviated significantly for only ≈1 min, yet their differential impact on pCREB extended over a much longer period, between 20 and 60 min and beyond, which is of likely significance for gene expression. The CaMK-dependent MAPK pathway may inform the nucleus about stimulus amplitude. In contrast, the CaMKIV pathway may be well suited to conveying information on the precise timing of localized synaptic stimuli, befitting its greater speed and sensitivity, whereas the previously described calcineurin pathway may carry information about stimulus duration.

The dynamic complexity of information processing within neuronal networks is greatly increased by activity-dependent changes in gene expression within individual neurons. Mammalian neurons express many tens of thousands of genes, several times more than any other known cell type. Alterations in the expression of these genes and in the state of the individual protein molecules they encode provide enormous capabilities for intracellular computation. Although little is known as yet about the extent to which such computation is actually used in the brain, understanding how various forms of neuronal activity control gene transcription is an obvious first step.

A leading paradigm of such regulation is the activation of the nuclear transcription factor CREB, the Ca^{2+}- and cAMP-responsive element binding protein (1). CREB becomes activated when phosphorylated on Ser-133 (2) and, through interaction with its nuclear partner CREB-binding protein (3), drives the transcription of a large number of genes. Although other residues can also be phosphorylated, with possible functional consequences (4, 5), Ser-133 has been the predominant focus of studies of transcriptional regulation. Ser-133 phosphorylation of CREB can be caused by electrical activity (6–8, 9, 10), neurotransmitter or hormone action on G-protein-coupled receptors (11, 12), or neurotrophin effects on receptor tyrosine kinases (13, 14). CREB has been strongly implicated in memory formation in a wide range of species (15–19). The richness of CREB signaling is greatly increased by its responsiveness to multiple intracellular signal transduction cascades. For example, direct activation of cAMP signaling (e.g., with forskolin (1, 8, 20), dopamine D1/D5 receptor stimulation (11), or cAMP analogs (21) gives rise to robust CREB phosphorylation. On the other hand, behavioral, synaptic or membrane-depolarizing stimuli chiefly recruit a calmodulin (CaM)/CaM kinase IV (CaMKIV)-mediated pathway (6, 7, 20, 22–24). In some cases, a Ras/mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK)-mediated pathway, modulated by protein kinase A (PKA) and protein kinase C, is also recruited (8, 21, 22, 25). The functional importance of these signaling cascades is not entirely clear. For example, it has been claimed that the CaMKIV pathway can be dispensable in hippocampal neurons, and that the Ras-ERK-Rsk2 pathway can be a predominant pathway to CREB phosphorylation (8). On the other hand, genetic deletion of CaMKIV greatly attenuated both basal and activity-dependent CREB phosphorylation in a wide variety of central neurons at time points after stimulation ranging from 2 min (24) to 45 min or more (23) in addition to blocking the activation of CREB-dependent gene expression (23). These studies raised fresh questions about the possible role of the MAPK pathway.

This paper describes experiments that focus on the kinetic contributions of various signaling pathways to the overall time course of CREB phosphorylation. The results provide information about how individual signaling pathways to CREB can be activated selectively and the possible functional significance of multiple converging pathways in carrying distinct information to the nucleus.

Experimental Procedures

Transfection and Pharmacology. Calcium phosphate transfections, immunocytochemistry, and culture of dissociated hippocampal

Abbreviations: CREB, Ca^{2+}- and cAMP-responsive element binding protein; pCREB, phosphorylated CREB; CaM, calmodulin; CaMK, CaM kinase; TTX, tetrodotoxin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAPK kinase; pMAPK, dually phosphorylated MAPK; PKA, protein kinase A; EGFP, enhanced green fluorescent protein.

‡To whom reprint requests should be addressed. E-mail: rwtsien@stanford.edu.

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CA3/CA1 pyramidal neurons were carried out as previously described (6, 7, 20). Before stimulation, neurons at 8–9 days *in vitro* were preincubated in 1 μM tetrodotoxin (TTX) for 1–2 h to block spontaneous neuronal activity; TTX was also present during the poststimulus phase. The usual stimulus (90 mM KCl in isotonic Tyrode's solution) clamps the membrane potential of the neurons close to 0 mV and, in light of known current–voltage relations, provides maximal activation of voltage-gated sources of calcium influx. TTX was typically not present during the 90 mM K^+ stimulus; as would be expected, inclusion of TTX during this type of stimulus has no effect (data not shown). However, for a 20 mM K^+ stimulus TTX was included, as this is a submaximal stimulus. Stimuli were applied by rapidly changing the perfusion solution over the coverslip substrate bearing the cultured neurons from control to high-K Tyrode's solution. Pharmacological agents were added 15–20 min before stimulation unless otherwise indicated and were present in the recovery solutions. Drugs were obtained from Calbiochem [TTX, KN-93, KN-92, KT 5720, R_P isomer of adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS), and forskolin] and Promega (U0126 and PD98059). The enhanced green fluorescent protein (EGFP) expression plasmid was obtained from CLONTECH; the expression plasmid for RasN17 was purchased from Upstate Biotechnology (Lake Placid, NY).

Imaging and Immunocytochemistry. Antibody to Ser-133-phosphorylated CREB (pCREB) (Upstate Biotechnology) was used at 1:500. Antibody to dually phosphorylated ERK1/2 was obtained from Promega or Upstate Biotechnology and used at 1:1000. Anti-MAP2 monoclonal antibody (Roche Molecular Biochemicals) was used at 1:500. Secondary antibodies were from Jackson ImmunoResearch. To image and quantify immunofluorescence, confocal microscopy was performed as previously described (6, 7, 20, 26). For purposes of quantification of dually phosphorylated MAPK (pMAPK), apical dendrites from all clearly identifiable individual neurons within randomly selected fields were included. Intracellular calcium imaging with fura-2 was conducted as previously described (20).

Results

Different Mechanisms Contribute to Early and Late Phases of CREB Phosphorylation. To distinguish the kinetics of different activity-dependent signaling pathways to CREB, we followed the time course of depolarization-induced CREB phosphorylation in the presence of different pharmacological inhibitors and under different stimulus conditions. First, PD98059, a MAPK kinase (MEK) inhibitor, was used to block the MAPK signaling pathway to CREB, and KN-93, an inhibitor of CaM-dependent kinases, was used to block the CaMK signaling pathway.

We provided a maximal depolarizing stimulus to the neurons (3 min of 90 mM K^+) and tracked the resulting dynamics of pCREB (Fig. 1). When neurons were fixed immediately after depolarization in the absence of pharmacological inhibition (Fig. 1*A Center*), robust formation of nuclear pCREB was detected with a phospho-specific antibody (shown in green). When instead fixation was delayed by a 60-min recovery period in control solution after stimulation, the majority of neurons still showed prominent nuclear pCREB, whereas approximately one-third of the cells had returned to their baseline state. This result is shown quantitatively in the intensity histograms below the images. During this recovery phase, nuclear pCREB intensity in individual neurons displayed an all-or-none character, as previously observed (7), suggesting that when CREB dephosphorylation occurs, it takes place rather abruptly and completely.

A very different pattern was observed when the MAPK pathway was inhibited with PD98059 (Fig. 1*B*). Whereas the formation of pCREB immediately after the K^+-depolarization was virtually unchanged by the MEK inhibitor, pCREB at the late time point was strongly reduced. Similar results were obtained with the distinct MEK inhibitor U0126 (data not shown). These data suggested that MAPK signaling played a selective role in the late phase of CREB phosphorylation. Yet another pattern of results was found in the presence of the potent CaMK inhibitor KN-93 (Fig. 1*C*). In this case, the immediate increase in pCREB was abolished, consistent with the known role of CaM kinases in the fast phase of depolarization-induced pCREB formation (6, 7, 20, 23, 24). Furthermore, the late phase was also reduced, as might be expected from the known requirement for CaM kinases in depolarization-activated MAPK pathway activation (26).

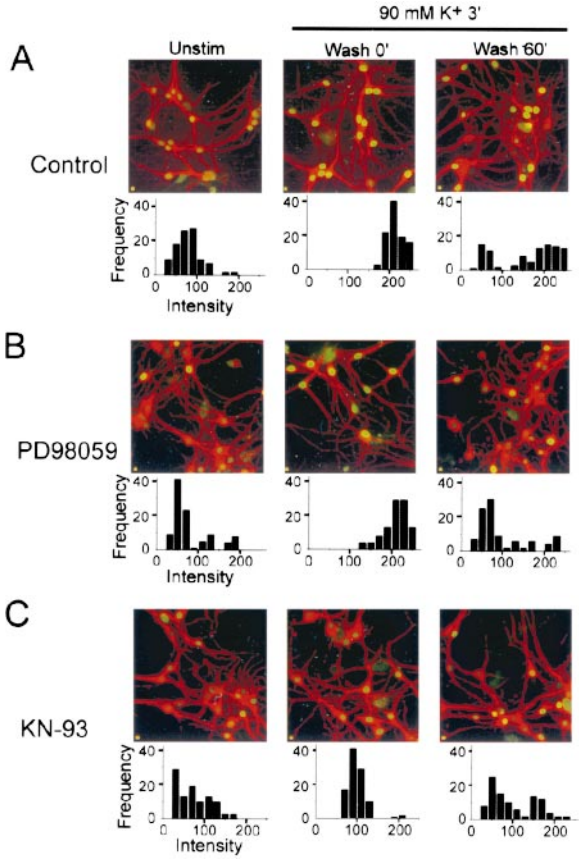

Fig. 1. CaMK inhibition and MAPK inhibition have distinct effects on the time course of CREB dephosphorylation. (*A*) Depolarization (90 mM K^+ for 3 min) induces rapid and stable CREB phosphorylation under control conditions in CA3/CA1 hippocampal pyramidal neurons. Cells are stained with anti-pCREB polyclonal antibody (green) and with anti-MAP2 monoclonal antibody (red) to identify neurons. Intensity histograms for pCREB are shown below each representative image. Note the all-or-none character of the pCREB staining during the recovery phase (wash 60′), as previously reported (7). (*B*) MEK inhibition with PD98059 (50 μM) selectively inhibits the late phase of CREB phosphorylation. (*C*) KN-93 (2 μM) abolishes the fast phase and reduces the slow phase of CREB phosphorylation. All results are representative of at least three independent experiments.

Fig. 2 shows pooled data from experiments of the kind illustrated in Fig. 1, detailing the time course of pCREB in the absence and presence of PD98059 and KN93. As in Fig. 1, it can be seen that the MAPK pathway is virtually without influence immediately after the 3-min depolarization but dominates at 60 min after the stimulation. The data at intermediate time points show that the influence of the MAPK pathway increases progressively with time. At 10 min after stimulation, removal of its contribution produces only a small effect, but by 30 min MAPK

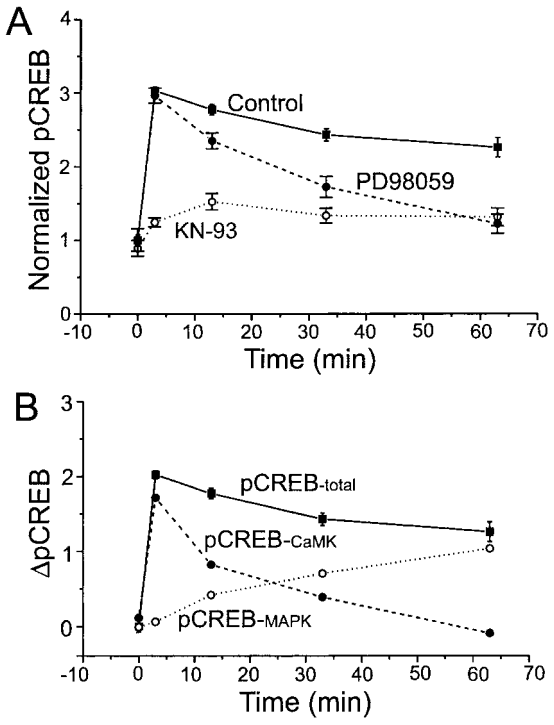

Fig. 2. Fast CaMK and slow MAPK pathways to pCREB. (*A*) Time course of the mean intensity of pCREB immunoreactivity under the conditions of Fig. 1. Values are normalized to baseline. (*B*) Calculated contributions of the MAPK pathway and MAPK-independent CaMK pathway to pCREB. The MAPK pathway contribution was taken as the difference between the control curve and the PD98059 curve in *A*. Note the slow rise of the pCREB-MAPK curve and the predominance of this pathway at 60 min. For isolating the pure CaMKIV pathway contribution, the KN-93 curve in *A* is understood to represent Control − MAPK contribution − MAPK-independent CaMK contribution. Because we have defined the MAPK contribution as Control − PD98059, the pure CaMKIV contribution can be represented as the difference between the PD98059 curve and the KN-93 curve (PD98059 − KN-93), as shown in *B*. Note the rapid rise and dominance of the CaMKIV pathway at early time points.

pathway inhibition ablates about half of the persistent nuclear pCREB.

Although KN-93 abolishes the fast phase virtually completely, a small, late signal could not be readily attributed to either pathway (≈20–30% of the total between 10 and 60 min; Fig. 2*A*), persisting even in the presence of KN-93. The persistence of this signal is to some extent consistent with an earlier report using a 10-min time point, in which neither KN-62 (a less potent CaMK inhibitor compared with KN-93) nor PD98059 alone could prevent depolarization-induced pCREB formation (22). The simplest explanation is incomplete kinase inhibition by the pharmacological agents, although an alternative candidate would be recruitment of some other kinase pathway (for example, a PKA-dependent pathway). However, although direct activation of cAMP/PKA signaling with forskolin can induce nuclear pCREB (1, 8, 20) [perhaps via MAPK (8, 25–28)], we have not observed any effect of PKA inhibitors on early or late depolarization-induced CREB phosphorylation (not shown). Therefore, although PKA pathways can contribute to CREB activation in various systems (when, for example, native noradrenergic, serotonergic, or dopaminergic pathways are able to contribute strongly), it appears that the CaMK and MAPK pathways are the dominant Ca^{2+}-dependent pathways to CREB in hippocampal pyramidal neurons.

Pharmacological Considerations. As with any pharmacological approach, it is important to review what is known about the actions of the inhibitors and how these factors might affect the analysis. The following considerations were taken into account. First, the MEK inhibitor PD98059 does not inhibit neuronal voltage-dependent Ca^{2+} influx (29). However, it can sharply reduce the excitability of hippocampal pyramidal neurons in response to repetitive electrical stimulation (30). We circumvented this potential complication by direct application of K^+-rich solution, bypassing intrinsic excitability mechanisms. Second, there are contradictory claims about whether PD98059 can inhibit CaM kinases (ref. 31, but see ref. 32). Such an effect is unlikely to be operational here, inasmuch as the effect of PD98059 is restricted to the late phase, unlike the action of KN-93, an agent known to block CaM kinases. Furthermore, U0126, a MEK inhibitor structurally and mechanistically distinct from PD98059, produced identical changes in the time course of pCREB, consistent with the idea that both agents blocked MAPK signaling selectively. Third, KN-93 can decrease Ca^{2+} influx (26, 29, 33). However, its structural analog KN-92 produces the same reduction of Ca^{2+} transients at a dose that does not inhibit CaM kinases or CREB phosphorylation, unlike KN-93 (7). We verified this observation by monitoring depolarization-induced Ca^{2+} elevations with fura-2 (26). At the dose used to block CREB phosphorylation, KN-93 reduced peak [Ca^{2+}] and total integrated [Ca^{2+}] by 22% and 38%, whereas KN-92 decreased the same parameters by 23% and 47%. Because KN-92 failed to block CREB phosphorylation, even at a higher concentration, the effects of the CaMK inhibitor KN-93 cannot be attributed to diminished Ca^{2+} entry. Furthermore, the findings with KN-93 are consistent with effects of CaMK inhibition by nonpharmacological approaches. These include the use of a CaM-inhibitory peptide introduced via patch pipette (6), antisense oligonucleotides directed against CaMKIV (7), a nuclear CaM-binding peptide introduced by neuronal transfection (20, 29), and the genetic deletion of CaMKIV (23, 24). Fourth, CaMKIV can powerfully stimulate the activity of a variety of MAPKs, including ERK (34). In hippocampal pyramidal neurons, CaMK activity is absolutely required for depolarization-induced activation of MAPK signaling, as assayed by immunocytochemical monitoring of dually phosphorylated ERK (26). Accordingly, KN-93 would be expected to inhibit downstream effects of both CaMK and MAPK signaling.

Contrasting Time Courses of pCREB Supported by Distinct Signaling Pathways. With these considerations in mind, we used the pooled data in Fig. 2*A* to derive the profiles of Ser-133 pCREB attributable to the MAPK and CaMK pathways (Fig. 2*B*). The time course of pCREB arising from MAPK was generated by subtracting the PD98059 data from the control data, which yielded a continuous slowly rising increase that continued to develop throughout the 1-h monitoring period (open circles). The component of pCREB strictly attributable to CaMK signaling and not MAPK signaling was derived by subtracting the KN-93 data from the PD98059 data. In this case, the level of pCREB rose immediately to a peak before declining steadily over the remainder of the period of observation (filled circles).

Blockade of Ras Signaling Spares Fast Rise pCREB but Selectively Inhibits the Slow Phase of pCREB Formation. We performed additional tests not involving pharmacological agents to verify that the Ras/MAPK pathway is specifically involved in late pCREB formation. It is well known that small GTPases such as Ras are critical in triggering MAPK pathway activation. In particular, Ras has been found to contribute to brain-derived neurotrophic factor-controlled CREB signaling in cortical neurons (14) and in depolarization-induced CREB phosphorylation in PC12 cells (35). To find out whether Ras plays a selective role in late CREB phosphorylation, we cotransfected hippocampal pyramidal cells with dominant-negative Ras (RasN17) along with EGFP as a

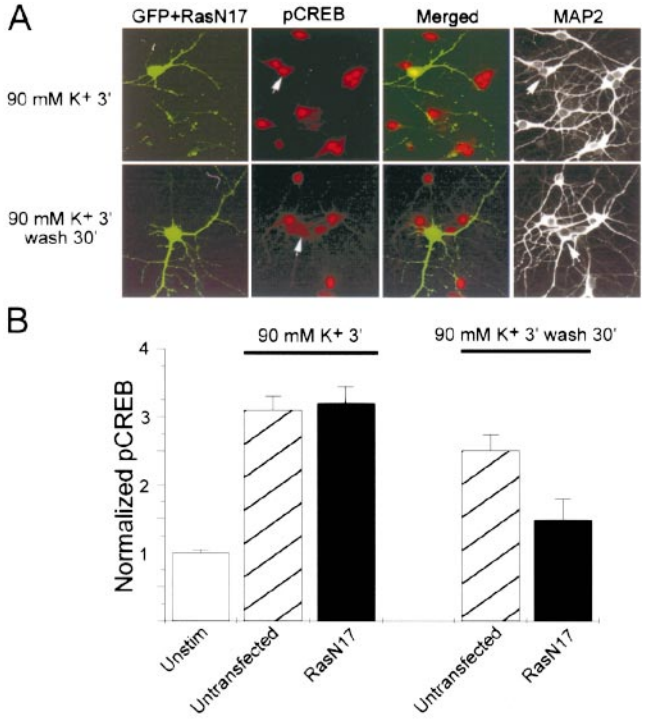

Fig. 3. Ras is selectively involved in the late phase of CREB phosphorylation. (*A*) Hippocampal pyramidal neurons from cultures 7 days *in vitro* were cotransfected with dominant-negative Ras (RasN17) and EGFP, then stimulated and fixed for immunocytochemistry 24 h later. pCREB staining is represented by red, EGFP marking the transfected cells is represented by green, and in the merged image, the conjunction of red and green appears as yellow. Note the robust CREB phosphorylation (yellow nucleus in the merged image) at the early time point (90 mM K^+ 3′) and the selectively reduced CREB phosphorylation at the late time point (wash 30′) in the transfected cells. MAP2 staining was used to identify all of the neurons; this is shown at the right in white. (*B*) Summary data are graphed below the representative examples. Dominant-negative Ras had no effect at the early time point ($P > 0.2$), but produced a partial inhibition at the late time point ($P < 0.01$), demonstrating that the MAPK pathway is selectively involved in the late phase but not the early phase of depolarization-induced CREB phosphorylation, consistent with the PD98059 results. Values are mean ± 1 SEM, $n > 50$ neurons for each condition.

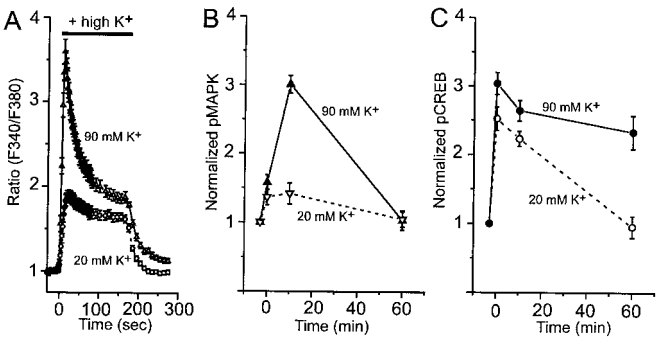

Fig. 4. Differential recruitment of the MAPK signaling pathway. (*A*) Dynamics of $[Ca^{2+}]_i$ generated by mild (20 mM K^+) or strong (90 mM K^+) depolarization. Traces represent somatic $[Ca^{2+}]_i$ measured with fura-2 ratiometric imaging. (*B*) Dynamics of pMAPK generated by mild (20 mM K^+) or strong (90 mM K^+) depolarization, determined with an antibody specific for pMAPK. (*C*) Dynamics of CREB phosphorylation generated by mild (20 mM K^+) or strong (90 mM K^+) depolarization.

marker (Fig. 3). The neurons were subjected to the standard 3-min K^+-depolarization, and early and late phases of depolarization-induced pCREB formation were examined in the transfected neurons. The increase in pCREB immunoreactivity immediately after the depolarizing stimulus was unaffected by RasN17 (representative images, Fig. 3*A Upper*; summary graph, Fig. 3*B Left*). However, transfection with the dominant-negative Ras significantly reduced the persistence of pCREB at 30 min in comparison with untransfected controls (Fig. 3*A Lower*; Fig. 3*B Right*). Relative to baseline, the inhibition was >50% ($P < 0.01$). These results were quantitatively indistinguishable from the results obtained with PD98059 (Fig. 2), providing further support for the conclusion that the Ras/MAPK pathway participates selectively in the slow phase of depolarization-induced CREB phosphorylation, not the fast phase.

A Stimulus That Poorly Activates MAPK Signaling Can Robustly Activate Fast Formation of pCREB. We conducted an additional nonpharmacological test that focused on the early phase of CREB phosphorylation. If, as we hypothesize, the MAPK pathway is involved in only the late phase of CREB phosphorylation, a stimulus that poorly recruits the MAPK pathway may selectively produce the immediate increase in pCREB but should fail to generate the late phase. The prediction was explored by examining the effects of a milder depolarizing stimulus, application of 20 mM K^+ solution. This solution is predicted to depolarize the neurons to approximately −40 mV, thereby causing a submaximal Ca^{2+} influx. Indeed, as indicated by Ca^{2+} fluorimetry with fura-2 (Fig. 4*A*), the 20 mM K^+ challenge gave rise to a much lower peak $[Ca^{2+}]_i$ than that generated by 90 mM K^+, although steady-state $[Ca^{2+}]_i$ levels were similar. Fig. 4*B* compares the effects of these stimuli on the activation of the MAPK pathway, monitored with an antibody specific for dually phosphorylated (activated) ERK as immunocytochemistry. In comparison with the strong ERK activation with the 90 mM K^+ depolarization, activated ERK is only weakly increased by the 20 mM K^+ challenge. The consequences of the two stimuli for CREB phosphorylation are illustrated in Fig. 4*C*. Whereas the rapid elevation in pCREB is virtually the same with the 20 mM K^+ stimulus as with the stronger stimulation, the increase of pCREB at 60 min is conspicuously absent. These data demonstrate that a stimulus that poorly activates pMAPK can generate robust early CREB phosphorylation while failing to support the late phase. Evidently, activation of MAP kinases requires a higher level of $[Ca^{2+}]_i$ than CaM kinases (Fig. 4 *A* and *B*). Rapid CaMK signaling to CREB is spared, but without substantial recruitment of MAPK signaling, the slower, sustained component of pCREB is lost (Fig. 4*C*).

Kinetic and Mechanistic Diversity of Signaling to CREB. Fig. 5 summarizes our conclusions, bringing together the data shown here with previous work. With membrane electrical activity, Ca^{2+} influx leads to the activation of at least three CaM-dependent signaling processes that converge on CREB Ser-133: first, a fast CaMKIV pathway mediated by rapid CaM translocation to the nucleus (20) and activation of a CaMK cascade (6), leading to CREB phosphorylation through CaMKIV (7); second, a slow CaMK/MAPK pathway, perhaps mediated by translocation of a Rsk or a MAPK (8, 14, 27) to the nucleus, leading to CREB phosphorylation through a kinase such as Rsk2; and third, a protein phosphatase-1 (PP1)-mediated pCREB dephosphorylation pathway promoted by calcineurin, which can be inactivated by prolonged stimulation (7, 11).

The speed of the CAMK pathway to CREB can be understood in part from its mechanism, which depends on the rapid nuclear translocation of CaM and rapid activation of intranuclear CaMKIV (7, 20). But why is the MAPK pathway to CREB slow? Is this property due to a slow nuclear translocation step or are the intrinsic dynamics of the MAPK activation cascade more likely to be limiting? Fig. 5*A* superimposes the kinetics of

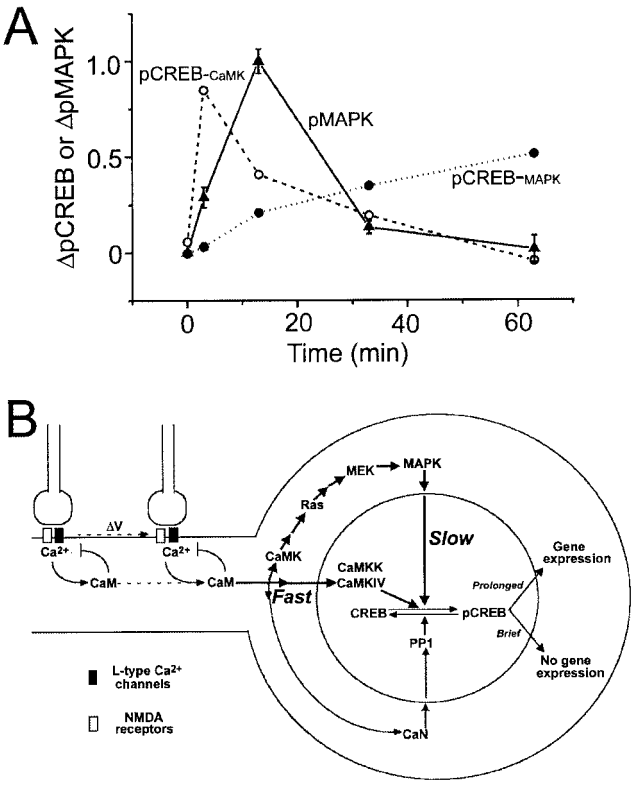

Fig. 5. Summary of depolarization-activated pathways to pCREB. (*A*) Superposed dynamics of 90 mM K^+-induced pMAPK (▲) compared with the pure CaMK contribution to pCREB (○) and the MAPK contribution to pCREB (●). Values are normalized against the maximal changes of pCREB or pMAPK. Note that the peak in pMAPK significantly lags behind the peak of pCREB-CaMK. Interestingly, a single strong membrane depolarization induced a transient increase in pMAPK, in contrast to the slow-developing persistent pCREB contributed by the MAPK pathway. (*B*) Model demonstrating the confluence of different depolarization-activated, CaM-dependent processes converging on CREB Ser-133 in hippocampal pyramidal neurons. Rapid calmodulin translocation to the nucleus controls rapid CaMKIV-dependent CREB phosphorylation, slow CaMK-dependent MAPK signaling controls a late phase of CREB phosphorylation, and a calcineurin (CaN)-dependent phosphatase (PP1) pathway modulates CREB dephosphorylation. Together, these processes control the dynamics of CREB phosphorylation and the stimulus-evoked gene expression decision. Potential interactions between the CaMK and MAPK pathways include direct activation of the MAPK pathway by CaMKIV.

pMAPK formation on the kinetics of the CaMK pathway to CREB and the MAPK pathway to CREB. Note that somatic/dendritic pMAPK formation is itself slow. With strong 90 mM K^+ stimulation, only a small increment in pMAPK formation is seen even at 3 min, and the peak is reached by 10–20 min; MAPK-dependent CREB phosphorylation follows this slow peak in pMAPK. In contrast, CaMK-dependent pCREB formation peaks much more rapidly, as previously shown, consistent with the faster operation of the CaMK pathway. Hence the slowness of the MAPK pathway to CREB is due in part to the fact that pMAPK formation is slow, perhaps because many intermediates lie upstream of pMAPK in the activity-dependent signaling cascade. Further delay may arise from a subsequent biochemical event. MAPK likely acts through a kinase such as Rsk2 to influence CREB, and factors controlling Rsk availability (such as nuclear translocation) could account for the further delay between pMAPK formation and MAPK-dependent pCREB formation (36, 37). Interestingly, we have found that the actual MAPK activation (ERK1/2 dual phosphorylation) induced by even the strong depolarization used here is transient and is virtually completely reversed by 30–60 min after stimulation (Fig. 5*A*). Therefore the late influence of the MAPK pathway on pCREB will likely be sensitive to phosphatase disruption, given the absence of continued upstream kinase stimulation. Calcineurin control of CREB-dependent gene expression (7, 11) could therefore be implemented through reversing phosphorylation mediated by either the CaMK or the MAPK pathway.

Discussion

It has been known for many years that several different protein kinases are capable of activating CREB through the phosphorylation of Ser-133. Our experiments provided insights into the functional consequences of this convergence. We found that activation of the Ras/MAPK pathway was specifically effective in promoting a slow phase of CREB phosphorylation. This component of Ca^{2+} entry-induced pCREB formation was eliminated by the MEK inhibitor PD98056, by dominant-negative Ras, and by reduction of the magnitude of peak Ca^{2+} entry. With each of these interventions, a rapidly developing but decaying component of pCREB formation was spared; this component has previously been associated with a kinase cascade involving CaM, CaMK kinase (CaMKK), and CaMKIV (6, 7, 20, 22, 29) and was ablated here by the inhibition of CaMK with KN-93. The intracellular Ca^{2+} signals that produced both rapid and slow components of pCREB formation or only the rapid component differed significantly for only ≈1 min, yet the consequences for pCREB extended over a much longer period, beginning at ≈20 min but lasting beyond 60 min. Because the duration of CREB phosphorylation is of demonstrated significance in determining whether transcription occurs, recruitment of the slower MAPK pathway to CREB phosphorylation could be strategically important, working together with regulation of CREB dephosphorylation through calcineurin (7, 11). In the following sections, we describe how these findings may help reconcile contradictory views of CREB regulation in recently published studies and discuss how the combination of multiple signaling pathways might be functionally advantageous for the precise control of gene expression.

Reconciling Evidence from Pharmacological and Knockout Approaches to CREB Regulation. Our observations may be relevant to conflicts in the literature about the relative importance of CaMK and MAPK pathways in controlling CREB phosphorylation and downstream transcriptional events. A number of investigations have implicated CaMKIV in rapid, activity-dependent CREB phosphorylation (6, 7, 20, 22, 29). However, it has been suggested (8) that Ras-ERK-Rsk2 can be a predominant pathway to Ser-133 pCREB, and that the CaMKIV pathway can be subsidiary or dispensable. Most recently, in two independent studies of CaMKIV mutant mice (23, 24), basal pCREB was greatly reduced in hippocampal, cerebellar, and cortical neurons, and activity-dependent rises in pCREB and c-fos expression were greatly attenuated. This response was found with direct stimulation as well as physiologic behavioral stimuli, including exposure to unfamiliar environments (24) and restraint stimulation (23), suggesting a dominant role for CaMKIV in pCREB formation by normal brain activity patterns. The CaMKIV knockout mice were deficient in pCREB at late time points as well as early ones (23), reopening questions about the relevance of MAPK signaling.

Our results with KN-93 show that inhibition of CaM kinases may exert an indirect effect on MAPK signaling, as well as directly blocking the CaMK kinase (CaMKK)/CaMKIV cascade. One possible explanation for CaMK control of MAPK signaling invokes a mechanism described at excitatory synapses. Cytoplasmic CaMKII can regulate MAPK signaling by phosphorylating synGAP, thereby disinhibiting Ras (38, 39). Perhaps

more relevant to the knockout experiments, the CaMKK/CaMKIV cascade can also powerfully stimulate MAPK activity (34), and both CaMKIV and the ERK1/2 MAP kinases are present in the nucleus and cytoplasm of hippocampal pyramidal cell (6, 20, 27). Thus, it remains possible that deletion of CaMKIV may have had multiple effects, not only removing the fast CREB phosphorylation pathway, but also depriving the slower MAPK pathway of necessary coactivation. In a test of this possibility, no difference in K^+-induced phosphorylation of ERK1/2 was seen in cortical neurons from CaMKIV knockout mice relative to wild-type neurons (23). However, it remains possible that the CaMKIV dependence of ERK1/2 activation was restricted to a neuronal subcompartment such as the nucleus, thus escaping detection by immunoblot of cortical cultures. Further experiments are needed to find out whether the MAPK pathway to pCREB remains intact in CaMKIV-deleted animals, now that we have a clearer idea of how that pathway may be recruited and observed. Regardless, it appears clear that CaMKIV plays a dominant role in CREB phosphorylation and CREB-dependent gene expression in the mammalian brain (23).

Functional Significance of Convergent Pathways for CREB Phosphorylation. There are interesting parallels between the present findings for CREB phosphorylation driven by Ca^{2+} entry and a previous study (14) on signaling mechanisms downstream of brain-derived neurotrophic factor. Finkbeiner *et al.* (14) provided clues that CaMKIV signaling was rapid (7) and MAPK signaling was slow and sustained, as quantitatively described here. What is the general significance of the convergence to a single regulatory site of multiple signaling pathways, each with its own distinct kinetics? Various interpretations are possible. The slowness of the MAPK pathway may not be optimized for a particular purpose but may simply be a natural consequence of incorporating many more intermediates than the fast CaMK pathway. According to this view, the MAPK cascade may be a generally useful pathway in a variety of cell types in which signaling speed is less important, whereas the CaMKK/CaMKIV pathway may represent a neuronal adaptation for rapid signaling. Another hypothesis is that the MAPK pathway is appropriately tuned to increase nuclear pCREB just when the influence of the convergent CaMKIV pathway is waning. Yet additional possibilities exist wherein the different kinetics of the CaMK and MAPK pathways are of computational significance: although activation of either pathway indicates the fact that some form of neuronal activity has occurred, each pathway might carry distinct information to the nucleus regarding the type, source, strength, or timing of the neuronal activity. For example, it has been hypothesized that the simultaneous triggering of a fast pathway and a slow pathway from one point in the cell may be informative about the distance from source to destination (14). Our observations suggest that the combination of CaMK and Ras/MAPK signaling is well suited for detection of stimulus strength. Milder Ca^{2+}-mobilizing stimuli would trigger the fast CaMK pathway alone, whereas stronger stimuli would additionally recruit the MAPK pathway and a more prolonged increase in pCREB. Prolongation of the stimulus would bring in additional regulation of dephosphorylation through shutting off of a calcineurin-protein phosphatase-1 dephosphorylation cascade (Fig. 5*B*) (7). Because CRE-mediated transcription is critically sensitive to the duration of the CREB phosphorylation, the interplay among these three pathways at ≈30 min after the stimulus represents a highly regulated control system—allowing amplitude information, perhaps from a particularly long or stressful stimulus, to critically tip the balance in favor of CREB-dependent gene expression.

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