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Multiple Ca^{2+} signaling pathways converge on CaM kinase in PC12 cells

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The role of multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) in mediating various Ca^{2+} signaling pathways was examined in PC12 cells. Conversion of the kinase to a Ca^{2+}-independent form was used to monitor which neurotransmitters activate the enzyme in situ. CaM kinase responds to Ca^{2+} influx elicited by ligand-gated Ca^{2+} channels for ATP and acetylcholine. It also responds to Ca^{2+} mobilization of IP_3-sensitive stores elicited by phospholipase C-linked receptors for ATP and acetylcholine as well as by caffeine. CaM kinase mediates the actions of many neurotransmitters and Ca^{2+} signaling pathways.

Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase); Calcium store; ATP receptor; Muscarinic receptor

1. INTRODUCTION

Many hormones, neurotransmitters and growth factors mediate their effects through an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Multiple pools of intracellular Ca^{2+} and multiple pathways of Ca^{2+} influx are involved [1]. A major cellular target of increased $[Ca^{2+}]_i$ is multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase), a ubiquitious protein kinase with a broad substrate specificity (reviewed in [2]). We wished to determine which receptors, acting via which pathways of Ca^{2+} signaling, are linked to activation of CaM kinase.

PC12 cells present a useful systen in which to study Ca^{2+} signaling, since these cells possess receptors for several neurotransmitters which increase $[Ca^{2+}]_i$ by different pathways. These cells respond to bradykinin, ATP, and acetylcholine, with activation of the phospholipase C-linked receptors B_2 [3], P_{2y} [4] and M_3 [5], respectively, and with the release of Ca^{2+} from an IP_3-sensitive store. In PC12 cells, this is the only rapidly exchanging Ca^{2+} store, and is responsive to both IP_3 and caffeine–ryanodine [6]. ATP and acetylcholine also stimulate Ca^{2+} influx by activation of the ligand-gated receptor-channel P_{2c} [4] and the nicotinic receptor [7], respectively. Stimulation of the latter receptors opens cation-selective channels that are part of the receptor complex and permits influx of extracellular Ca^{2+} and Na^+. Additional Ca^{2+} may subsequently enter due to the resultant depolarization of the cells and opening of voltage-sensitive Ca^{2+} channels [7].

We used the Ca^{2+}-dependent autophosphorylation of CaM kinase as a biochemical Ca^{2+} sensor, and asked which receptors acting through which of the Ca^{2+} signaling pathways, activate the enzyme in PC12 cells. By analogy to use of protein kinase C translocation as an assay of its in situ activation we used the conversion of CaM kinase to its Ca^{2+}-independent form to examine its response to cellular stimuli.

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2. MATERIALS AND METHODS

PC12 cells were kindly provided by Dr. Eric Shooter (Stanford University). Cells were grown in DME medium with 5% defined/supplemented calf serum and 10% horse serum (HyClone Laboratories) in a humidified incubator containing 10% CO_2 and 90% air at 37°C. Determination of cytosolic free Ca^{2+} in cell populations was performed at 30°C using Indo-1 (Molecular Probes), as previously described [8]. Autonomous activity of CaM kinase was assayed using the peptide substrate autocamtide-2, as described [9]. All other reagents were from Sigma Chemical Co. unless noted otherwise.

3. RESULTS

We first tested whether CaM kinase responds to stimuli which elicit an influx of extracellular Ca^{2+}. PC12 cells were treated with 100 μM ATP or 100 μM 1,1-dimethyl-4-phenylpiperazinium (DMPP, a nicotinic receptor agonist), and activation of CaM kinase was monitored by assaying its Ca^{2+}-independent or autonomous activity (Fig. 1). ATP increased $[Ca^{2+}]_i$ and elevated autonomous CaM kinase activity from $3.5 \pm 0.5\%$ to $15.4 \pm 1.3\%$ of maximal Ca^{2+}-stimulated activity within 10 s. The effect of ATP was largely dependent on extracellular Ca^{2+} (Fig. 1). Stimulation of the nicotinic receptor in PC12 cells is also coupled to activation of CaM kinase. Ca^{2+}-independent activity increased to $14.0 \pm 1.3\%$ of maximal activity within 10 s of DMPP treatment. This effect was entirely dependent on ex-

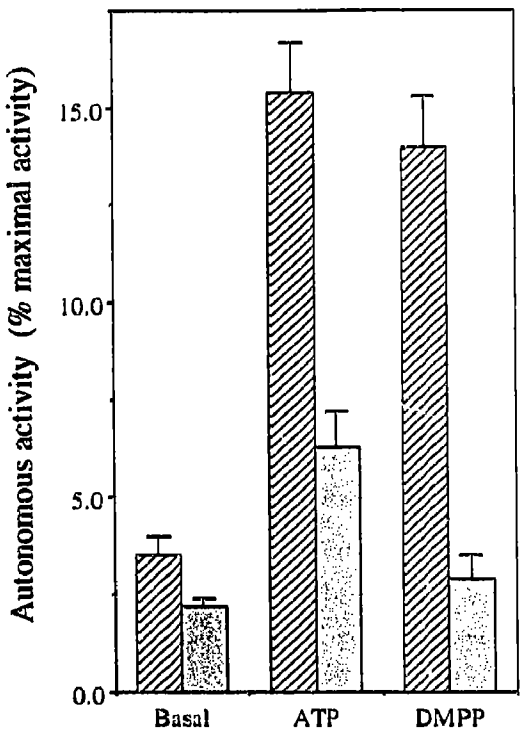

Fig. 1. Activation of CaM kinase by ATP or DMPP is dependent on extracellular Ca^{2+}. PC12 cells were stimulated by the addition of 100 μM ATP or 100 μM DMPP to the growth medium. After 10 s the cells were homogenized, and both the Ca^{2+}-independent and Ca^{2+}-dependent (maximal) CaM kinase activity was determined, using autocamtide-2 as substrate. Autonomous activity is given as the Ca^{2+}-independent activity as a percentage of maximal Ca^{2+}-stimulated activity. Maximal activity did not vary in response to stimulation and is defined as 100%. Slashed bars indicate autonomous activity in the presence of extracellular Ca^{2+}, and shaded bars indicate autonomous activity in cells that were treated with 10 mM EGTA for 10 s prior to addition of ATP or DMPP. Basal levels of autonomy in the presence and absence of extracellular Ca^{2+} is shown for comparison. Data is averaged from four experiments and error bars indicate the standard error of the mean (S.E.M.).

tracellular Ca^{2+} (Fig. 1), and was not inhibited by blockade of voltage-sensitive Ca^{2+} channels (data not shown).

We monitored changes in $[Ca^{2+}]_i$ during stimulation of PC12 cells to determine whether influx of Ca^{2+} through the ATP- and DMPP-ligated Ca^{2+} channels was responsible for activation of CaM kinase. The predominant effect of ATP treatment is Ca^{2+} influx (Fig. 2A). This accounts for most of the conversion of CaM kinase to its autonomous form (Fig. 1). The small transient increase in $[Ca^{2+}]_i$ seen in the absence of extracellular Ca^{2+} is likely due to release of Ca^{2+} from IP_3-sensitive stores by stimulation of the P_{2y} receptor for ATP [4]. This small increase in $[Ca^{2+}]_i$ parallels the partial activation of CaM kinase observed under these conditions (Fig. 1). By contrast, DMPP was completely dependent on extracellular Ca^{2+} for increasing $[Ca^{2+}]_i$ (Fig. 2B), a property that paralleled its effect on activation of CaM kinase (Fig. 1). Thus, CaM kinase is tightly coupled to Ca^{2+} influx through ATP- and acetylcholine-liganded receptor-channels, and may thereby mediate some of the actions of these transmitters.

We have previously shown that CaM kinase responds to mobilization of an intracellular Ca^{2+} store by bradykinin [8]. Does this extend to other receptors that mobilize intracellular Ca^{2+} and does release of Ca^{2+} from the single IP_3- and caffeine-sensitive Ca^{2+} store in PC12 cells activate CaM kinase? Stimulation of the M3 muscarinic receptor with 1 mM muscarine activated CaM kinase to 14.4 ± 0.7% of maximal activity (Fig. 3). This initial activation (10 s) is due almost entirely to release of an intracellular Ca^{2+} pool, since the same level of CaM kinase activation and the same initial rise in $[Ca^{2+}]_i$ was seen in the presence or absence of extracellular Ca^{2+} (Figs. 3 and 4A). Influx of extracellular Ca^{2+} is necessary for maintainence of $[Ca^{2+}]_i$ levels at later time points (60 s). This Ca^{2+} influx is also critical or sustained activation of CaM kinase, since its activity returns to basal levels within 60 s in the absence of extracellular Ca^{2+}. Thus, three distinct receptors on PC12 cells, P_{2y} (for ATP), M_3 (for acetylcholine) and B_2 (for bradykinin), can activate CaM kinase by mobilization of intracellular Ca^{2+}.

The finding that PC12 cells possess a single, rapidly exchangeable Ca^{2+} store, and that this store is responsive to caffeine as well as IP_3 [6], enabled us to determine whether this IP_3- and caffeine-sensitive store is responsible for activation of CaM kinase. Release of Ca^{2+} from the IP_3-sensitive store was stimulated with 10 mM caffeine in the absence of extracellular Ca^{2+}. Autonomous CaM kinase activity increased to 13.9 ± 2.1% within 10

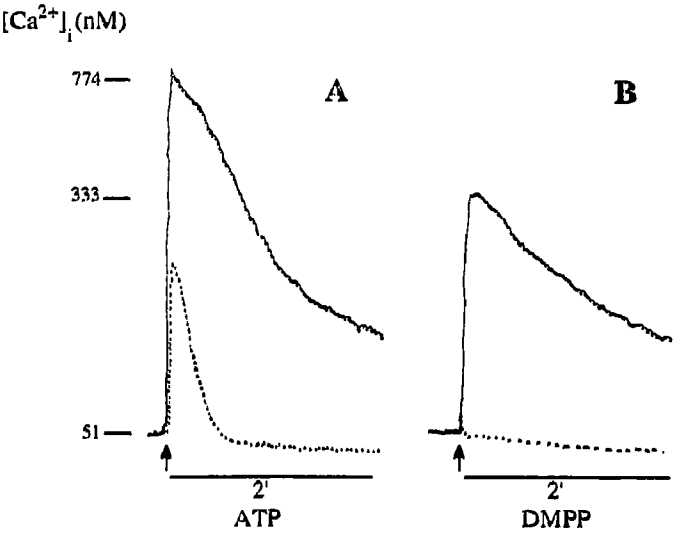

Fig. 2. ATP- and DMPP-stimulated increases in $[Ca^{2+}]_i$ are dependent on extracellular Ca^{2+}. Indo-1-loaded PC12 cells were stimulated by the addition of 100 μM ATP or 100 μM DMPP (arrows). Intracellular free Ca^{2+} was determined by Indo-1 fluorescence as described [6]. Dashed traces indicate $[Ca^{2+}]_i$ in cells treated with 10 mM EGTA prior to the addition of ATP or DMPP to reduce extracellular Ca^{2+}. Single traces are shown which are representative of three similar experiments.

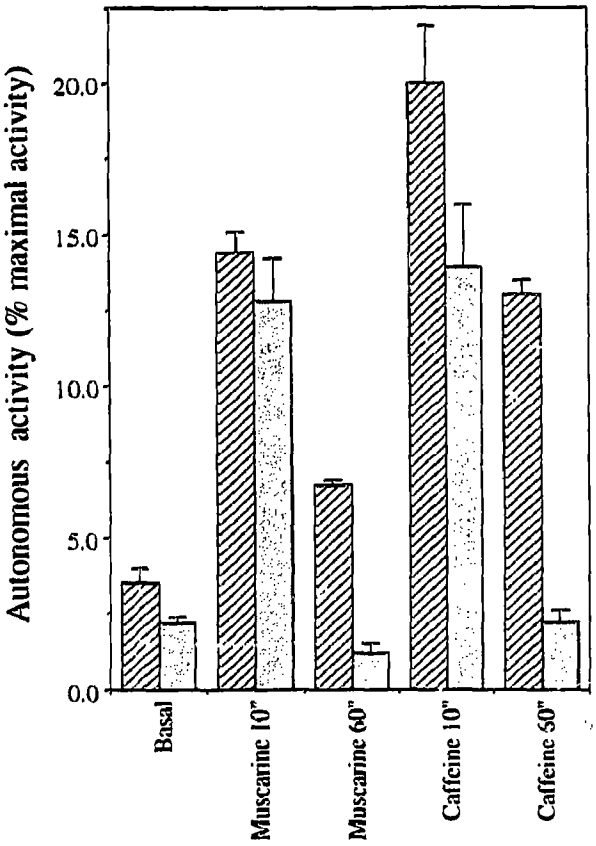

Fig. 3. Muscarine and caffeine activate CaM kinase by mobilization of $[Ca^{2+}]_i$. PC12 cells were stimulated by 1 mM muscarine or 10 mM caffeine in the presence (slashed bars) or absence (shaded bars) of extracellular Ca^{2+}, and CaM kinase autonomous activity was determined at the times indicated, as described in Fig. 1.

s and declined to basal levels by 60 s (Fig. 3). This indicates that release of Ca^{2+} from the IP_3-sensitive store is, indeed, functionally coupled to activation of this kinase. Treatment with caffeine in the presence of extracellular Ca^{2+} produced a more sustained activation of CaM kinase. Caffeine increased $[Ca^{2+}]_i$ transiently in the absence of extracellular Ca^{2+} and sustained elevated $[Ca^{2+}]_i$ longer when extracellular Ca^{2+} was available (Fig. 4B). Maintenance of CaM kinase activity by both muscarine and caffeine in the presence, but not absence, of extracellular Ca^{2+} suggests that influx of Ca^{2+}, subsequent to depletion of the IP_3- and caffeine-sensitive store, is important for mediating the physiological actions of these agents.

4. DISCUSSION

Localization of CaM kinase in cytosolic, cytoskeletal, and membrane fractions, of a variety of cell types, and its additional localization on postsynaptic densities in neurons, positions it to respond to changes in $[Ca^{2+}]_i$ throughout the cell. We have demonstrated previously

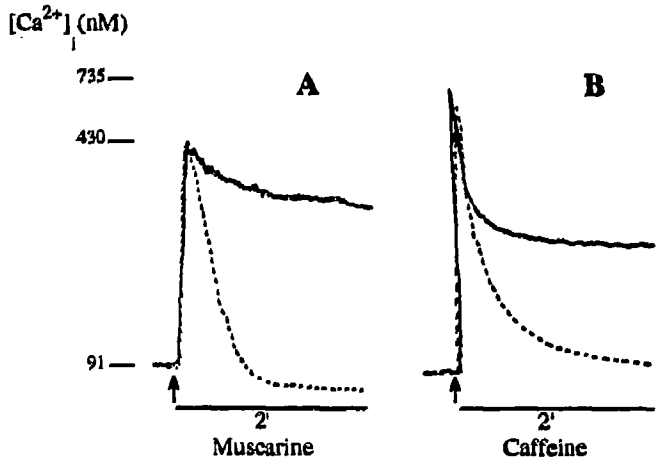

Fig. 4. Muscarine and caffeine mobilize intracellular stores of Ca^{2+} and stimulate Ca^{2+} influx. Indo-1-loaded PC12 cells were stimulated by (A) 1 mM muscarine or (B) 10 mM caffeine at the time indicated by arrows, and $[Ca^{2+}]_i$ was determined, as described, in the presence (solid traces) or absence (dashed traces) of extracellular Ca^{2+} as described in Fig. 2.

that both K^+ depolarization, which causes an influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels, and bradykinin, which releases Ca^{2+} from an intracellular store, activate CaM kinase in PC12 cells. We show here that a variety of agonists, which use different mechanisms to increase $[Ca^{2+}]_i$ at specific cellular locations, all converge on the activation of CaM kinase. This, along with the wide substrate specificity of the enzyme, allows CaM kinase to mediate the action of a wide array of receptors and pathways of Ca^{2+} signaling on diverse substrates through the cell.

The choice of Ca^{2+} signaling pathway may determine which Ca^{2+}-sensitive process will be modulated. For example, secretion of catecholamines is stimulated by Ca^{2+} influx that produces high local $[Ca^{2+}]_i$ at the plasma membrane, but not by treatments that produce a dispersed rise in $[Ca^{2+}]_i$ [10]. In PC12 cells, DMPP acting at nicotinic receptors and ATP acting at P_{2c} receptors stimulate Ca^{2+} influx that may primarily affect CaM kinase at or near the plasma membrane. Such localized activation may underlie the ability of CaM kinase to modulate release of neurotransmitter at nerve terminals in neurons and at vesicle release sites in PC12 cells [11].

The IP_3-sensitive stores in PC12 cells appear to be localized throughout the cytosol [12], where much of CaM kinase and many of its substrates are localized [2]. We show that at least three receptors in PC12 cells that couple to intracellular mobilization of Ca^{2+} (P_{2y}, M_3, and B_2, for ATP, acetylcholine, and bradykinin, respectively) all activate CaM kinase. Furthermore, we took advantage of the fact that in PC12 cells caffeine releases Ca^{2+} from the IP_3-sensitive store to show that it is the release of Ca^{2+} from this specific store which activates CaM kinase. Hormones and neurotransmitters that sig-

nal via this pathway may be coupled to the dispersed activation of cytosolic CaM kinase and to the phosphorylation and regulation of its many cytosolic substrates.

Our in situ findings differ from the in vitro biochemical characteristics of CaM kinase in two ways. First, only 20% of the kinase becomes autonomous after various physiological stimuli (Figs. 1 and 3) even though in vitro autophosphorylation can make it 70–80% autonomous. It is possible that each Ca^{2+} signaling pathway only has access to a subpopulation of CaM kinase molecules in the cell. Alternatively, calmodulin may be limiting so that submaximal stimulation is obtained. This may reflect the fact that there are many calmodulin-binding proteins and that CaM kinase has a relatively low affinity for calmodulin. In fact, we have recently shown that stimulation of CaM kinase in PC12 cells can be enhanced by release of bound calmodulin [9]. Second, the inhibitory phase of autophosphorylation that blocks binding of calmodulin is seen in vitro (reviewed in [2]) but is not seen after in situ stimulation. We have recently found that dissociation of calmodulin from an autophosphorylated kinase takes many seconds after Ca^{2+} levels are lowered below threshold values for CaM kinase activation [13]. This trapped state, in which calmodulin is still bound to the enzyme, is likely to be prolonged following stimuli in which $[Ca^{2+}]_i$ declines slowly (Figs. 2A and 4A; [8,13]). The lack of inhibitory phosphorylation in situ may arise by the trapping of calmodulin, which would prevent the inhibitory phosphorylation of the calmodulin-binding domain.

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