Activation of a Calcium-Calmodulin-dependent Protein Kinase I Cascade in PC12 Cells*

(Received for publication, April 26, 1996, and in revised form, June 28, 1996)

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It has been observed that the activity of Ca\textsuperscript{2+}-calmodulin (CaM)-dependent protein kinase I is enhanced up to 50-fold by its phosphorylation in vitro by a distinct CaM kinase I kinase (Lee, J. C., and Edelman, A. M. (1994) J. Biol. Chem. 269, 2158-2164). It has, however, been unclear whether this event represents an acute form of cellular regulation. We demonstrate here the phosphorylation and activation of CaM kinase I in PC12 pheochromocytoma cells in response to elevation of intracellular Ca\textsuperscript{2+}. Treatment of PC12 cells with the Ca\textsuperscript{2+}-ionophore, ionomycin, or with a depolarizing concentration of KCl, led to rapid, biphasic phosphorylation of CaM kinase I and to increased in CaM kinase I activity of 5.1- and 7.3-fold, respectively. Depolarization-induced activation of CaM kinase I was reduced by ~80% by blockade of Ca\textsuperscript{2+} influx through L-type voltage-dependent Ca\textsuperscript{2+} channels and completely abolished by removal of extracellular Ca\textsuperscript{2+}. The ability of PC12 cell CaM kinase I to be phosphorylated and activated by purified CaM kinase I kinase in vitro was markedly reduced by prior depolarization of the cells, consistent with intracellular phosphorylation and activation of CaM kinase I by CaM kinase I kinase. These results demonstrate the existence in PC12 cells of a CaM kinase I cascade, the function of which may be to sensitize cells to signal-induced elevations of intracellular Ca\textsuperscript{2+}.

Control of cellular function by extracellular signal-induced elevation of intracellular Ca\textsuperscript{2+} is a common theme in biological systems (1). In many cases, the Ca\textsuperscript{2+} signal is delivered to the appropriate intracellular target protein via phosphorylation catalyzed by a Ca\textsuperscript{2+}-calmodulin (CaM)-dependent protein kinase (CaMK) (2, 3). The roles of some members of the CaMK group in specific Ca\textsuperscript{2+}-regulated physiological processes have been clearly defined, as exemplified by the involvement of myosin light chain kinase and phosphorylase kinase in Ca\textsuperscript{2+}-dependent regulation of muscle contraction and glycolysis, respectively. By contrast, the Ca\textsuperscript{2+}-dependent cellular events in which CaMKI, a more recently characterized member of this group (reviewed in Ref. 4), participates are currently unknown. However, since CaMKI exhibits a broad tissue and phylogenetic distribution (5–8) as well as the ability to phosphorylate in vitro a variety of synthetic peptides (9, 10), and proteins such as synapsins I and II (5, 11, 12), the cystic fibrosis transmembrane conductance regulator (13), and cyclic AMP response element binding protein (14), it appears likely to mediate an array of Ca\textsuperscript{2+}-regulated cellular functions in diverse tissues and cell types. Recently, activating transcription factor-1, another member of the CaMK family, has been shown to be phosphorylated and activated by CaMKI, suggesting that one important role of CaMKI may be to mediate Ca\textsuperscript{2+}-dependent transcriptional activation (15).

CaMKI exhibits complex regulation by Ca\textsuperscript{2+}-CaM. On the one hand, Ca\textsuperscript{2+}-CaM directly activates CaMKI through relief of intrasteric autoinhibition (7, 16). In addition, CaMKI is strongly dependent for its activity upon its Ca\textsuperscript{2+}-CaM-dependent phosphorylation by a kinase, CaMKI kinase (CaMKIK) (7, 17–21). Activation results from the phosphorylation by CaMKIK of a single residue, Thr-177 (7, 20, 21) located in the "activation loop" of CaMKI (22). This residue is at a position equivalent to the activating phosphorylation sites in other CaMK group members. The CaMKI kinase, CaMKIK (CaMKIK), is a protein kinase (2, 3), and is strongly dependent for its activity upon its Ca\textsuperscript{2+}-CaM-dependent phosphorylation by a kinase, CaMKII kinase (CaMKIK) (7, 17–21). Activation results from the phosphorylation by CaMKIK of a single residue, Thr-177 (7, 20, 21) located in the "activation loop" of CaMKI (22). This residue is at a position equivalent to the activating phosphorylation sites in other CaMK group members.

While these data predict that the phosphorylation and activation of CaMKI by CaMKIK may occur in vivo, there has, to date, been no experimental evidence for the existence of such a cascade in living cells. Furthermore, it is unclear whether such a phosphorylation would be constitutive or dependent upon cell-signaling events. We report here, using the PC12 pheochromocytoma cell line, that a CaMKI cascade exists in living cells and that initiation of this cascade can occur in response to either a Ca\textsuperscript{2+}-ionophore, ionomycin, or to a membrane depolarization-induced Ca\textsuperscript{2+} influx through L-type voltage-dependent Ca\textsuperscript{2+} channels.

EXPERIMENTAL PROCEDURES

Materials—D600 was obtained from Knoll, A.G. Ionomycin and okadaic acid were from Calbiochem. [γ-32P]ATP and [32P]orthophosphate (P\textsuperscript{32}i) were purchased from DuPont NEN. Synapsin site 1 peptide was synthesized by the Biomedical Research Core Facilities of the University of Michigan. All other chemicals were of reagent grade or better and purchased from standard suppliers.

Proteins and Immunological Reagents—Calmodulin was obtained from Boehringer Mannheim. CaMKIK was purified from pig (17, 30), or rat (21), brain as described previously. The rat brain CaMKIK preparation used in this study was an approximately equal mixture of the α and β isoforms, both of which activate CaMKI by phosphorylating Thr-177 (21). Antibody CC76 is a polyclonal antiserum prepared by injection into rabbits of expressed rat CaMKI, and determined to be CaMKI-specific by Western blotting of PC12 cells, and a variety of other tissues and cell types (8). Serum from immunized rabbits (nonimmune serum) was obtained from Cappel. Protein A-Sepharose was purchased from Pharmacia Biotech Inc. Donor horse serum was obtained from JRH Biosciences.
Agonist Treatment of Cells—Stock cultures of PC12 cells were maintained as described previously (31). Experimental cultures were placed in RPMI medium plus 1% horse serum 18 to 24 h prior to drug or KCl treatment. Replicate cultures were treated with agonists for the time periods and at the concentrations indicated in the figure legends. D600 was dissolved in deionized water as a 100× stock solution, and ionomycin was prepared as a 500× solution in dimethyl sulfoxide. Depolarization of intact cells was accomplished by raising extracellular K+ to 40 mM by addition of an appropriate volume of 150 mMKCl in deionized water. For PC12 cell phosphorylation experiments (see Fig. 1), agonist treatment occurred during the final 5–60 min of a 2-h period of metabolic radiolabeling in Krebs-Ringer’s HEPES-buffered saline containing 300 μCi/ml 32P.

Immunoprecipitation—For determination of CaMKI phosphorylation in PC12 cells, cultures were rinsed with PBS (35°C), and cellular lysates were prepared as described previously (32) under either denaturing or nondenaturing conditions. For the former, cells were lysed in 1% SDS in PBS for 5 min at 95°C. Lysates were then diluted 10-fold with IP buffer (% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 25 mM NaF, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 60 kallikrein inactivating units of aprotinin/ml, 100 mM dithiothreitol, and 1 mM sodium orthovanadate). For the latter, lysis was with IP buffer for 10 min at 4°C. Lysates prepared by either method were preclarified with 6 mg of protein A-Sepharose, and volumes containing equal trichloroacetic acid-precipitable cpm (typically 1–3×106) were incubated for 1 h with 1 μl of either CC76 or nonimmune serum. Immune complexes were captured with 3 μg of protein A-Sepharose, washed twice with 1 ml of IP buffer, and then twice with 1 ml of 50 mM Tris-HCl, pH 7.6. Assay of the CaMKI activity of the immune complexes was performed immediately.

Immune Complex Assay of CaMKI Activity—Immune complexes were resuspended in an assay mixture consisting of: 50 mM Tris, pH 7.6, 0.5 mM dithiothreitol, 0.5 mg of bovine serum albumin/ml, 1 mM CaCl2, 1 μM CaM, 10 mM MgCl2, 200 μM [γ-32P]ATP (~100 cpm/pmol), and 50 mM synapsin site 1 peptide in a final volume of 76 μl. The assay mixture was incubated at 30°C for 2.5 and 5 min at which times 20-μl aliquots were removed and 32P incorporation into synapsin site 1 peptide was quantified by phosphocellulose adsorption and scintillation counting as described previously (11).

RESULTS

Elevation of Intracellular Ca2+ Leads to Phosphorylation of CaMKI in PC12 Cells—To assess whether CaMKI is phosphorylated in response to agonists which elevate intracellular Ca2+, PC12 cells, incubated with 32Pi, were treated with either a depolarizing concentration of KCl (40 mM) or with the Ca2+-ionophore, ionomycin (2 μM). CaMKI was then specifically immunoprecipitated from cellular lysates, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1A, elevation of intracellular Ca2+ induced by either membrane depolarization or ionomycin resulted in increased CaMKI phosphorylation. Phosphorylation was rapid in response to either treatment with a peak of 32P incorporation reached within ~3 min (Fig. 1B). As quantified by scanning densitometry of the autoradiographic images, the maximal increase in 32P incorporation (relative to nontreated cells), was 5.3- and 6.2-fold after treatment for 3 min with KCl and ionomycin, respectively (Fig. 1C). The continued presence of agonist for extended periods of time (15 min for ionomycin, 15–60 min for KCl) led to a reduced level of incorporation suggesting that with time, the rate of dephosphorylation of CaMKI may be enhanced.

Previous studies have suggested the existence of isoforms of CaMKI (5, 11, 12). The predominant form in PC12 cells migrates with approximately the same electrophoretic mobility as the ovalbumin M, ~43,000 standard (8). This form appears to correspond to the α form of CaMKI isolated from rat brain (11, 12). In the gel system used here, it electrophoreses as a tightly spaced doublet (Fig. 1). Depending on the conditions of SDS-PAGE (e.g., gel length), such a doublet is also observed in purified preparations of CaMKIα (data not shown). As shown in Fig. 1, both bands of the doublet incorporate 32P. Thus, it is possible that the appearance of a doublet may be due to factor(s) other than phosphorylation, for example amino acid sequence differences or a different post-translational modifica-
Elevation of Intracellular Ca\(^{2+}\) Leads to Activation of CaMKI in PC12 Cells—Based on previous reports that CaMKI is phosphorylated and activated by purified CaMKIK in vitro (7, 17-21), we asked whether the Ca\(^{2+}\)-induced intracellular phosphorylation of CaMKI illustrated in Fig. 1 is accompanied by its activation. As shown in Fig. 2A, elevation of intracellular Ca\(^{2+}\) by treatment for 5 min with either 40 mM KCl or 2 \(\mu M\) ionomycin led to increases in CaMKI activity relative to nontreated cells of 7.3-fold and 5.1-fold, respectively. Similar increases in CaMKI activity were observed in 15 independent experiments with KCl and 7 independent experiments with ionomycin. The basis for the conclusion that these increases reflect the specific activation of PC12 cell CaMKI, without contribution of either CaMKII or CaMKIV to the observed activity, is as follows. (i) A synthetic peptide, synapsin site 1 peptide (11), modeled on the site phosphorylated in synapsin by CaMKI (33) was used as substrate. Although an effective substrate for CaMKI, this peptide is poorly phosphorylated by CaMKII (12). (ii) CaMKI activity was measured in immune complexes after immunoprecipitation with an antibody (CC76) directed against recombinant expressed rat CaMKI (6). This antibody specifically recognizes CaMKI in a variety of cells and tissues, including PC12 cells (8). (iii) In each assay, a replicate culture was included in which immunoprecipitation was performed with nonimmune serum substituted for CC76. Since the former does not immunoprecipitate CaMKI (see Fig. 1), it was treated as a nonspecific “blank” and subtracted from values obtained using CC76. (iv) As shown in Fig. 2A, CaMKI immunoprecipitated from nontreated cells is activated 7.4-fold by its regulatory kinase kinase, CaMKIK added in vitro, whereas the nonspecific activity is unresponsive to added CaMKIK. (v) Finally, CaMKI immunoprecipitated from either nontreated or depolarized cells was completely dependent for its activity upon the presence in the assay mixture of Ca\(^{2+}\)-CaM (data not shown). By contrast, CaMKII and CaMKIV acquire Ca\(^{2+}\)-CaM-independent activity after phosphorylation in vitro (30, 34-39) or in intact cells (40, 41).

To further explore the mechanism of Ca\(^{2+}\)-induced CaMKI phosphorylation and activation, we added purified rat brain CaMKIK to CaMKI immunoprecipitated from lysates of depolarized or ionomycin-treated cells. In contrast to the 7.4-fold activation by CaMKIK of CaMKI from nontreated cells, CaMKI from either depolarized or ionomycin-treated cells was minimally activated by CaMKIK (1.3- and 1.6-fold, respectively) (Fig. 2A). This lack of additivity is consistent with the KCl and ionomycin stimulation of CaMKI activity being mediated by the same mechanism as CaMKIK-induced activation. In two independent experiments using pig brain CaMKIK (17), a similar nonadditivity was also observed. In a similar fashion, the ability of immunoprecipitated CaMKI to be phosphorylated by purified CaMKIK in vitro was markedly attenuated by prior KCl treatment of the cells (Fig. 2B). Since the phosphorylation site responsible for the activation of CaMKI has been identified as Thr-177 (7, 20, 21), these data indicate that Ca\(^{2+}\) elevation leads to the phosphorylation of Thr-177 of CaMKI intracellularly, thereby rendering the enzyme less responsive to activating phosphorylation by CaMKIK subsequently added in vitro.

From the results shown in Figs. 1 and 2, it is also clear that CaMKI from nontreated cells exhibits a slight, but detectable, level of phosphorylation and activity. Moreover, whereas CaMKI immunoprecipitated from nontreated cells was acti-
serum-starved for 3 h. Both groups of cells were then either nontreated or depolarized with KCl for 5 min. The withdrawal of serum had no appreciable effect on the activity of CaMKI immunoprecipitated from depolarized cells (42.7 ± 4.5 pmol/min in the serum-starved condition versus 38.0 ± 2.3 pmol/min in the 1% serum condition; mean ± S.E., 2 experiments). By contrast, serum withdrawal led to an ~58% decrease in the activity of CaMKI immunoprecipitated from nontreated cells (4.2 ± 0.2 pmol/min in the serum-starved condition versus 9.9 ± 0.7 pmol/min in the 1% serum condition; mean ± S.E., 2 experiments). The net effect was that the response of CaMKI activity to depolarization was sensitized by serum withdrawal (10.4-fold activation in the serum-starved condition versus 3.9-fold in the 1% serum condition, in this set of experiments). These data are consistent with a serum component(s) inducing a small elevation of intracellular Ca\(^{2+}\) leading to partial phosphorylation and a slight elevation of CaMKI activity in nontreated cells.

Depolarization-induced CaMKI Activation Requires Influx of Extracellular Ca\(^{2+}\)—The results of Figs. 1 and 2 indicate that increases in intracellular Ca\(^{2+}\) can initiate the phosphorylation and activation of CaMKI in intact PC12 cells, i.e. can initiate a CaMKI cascade. To identify the source(s) of Ca\(^{2+}\) driving this cascade, we determined the effect of the manipulation of Ca\(^{2+}\) influx upon CaMKI activation. As shown in Fig. 3A, D600, an inhibitor of L-type voltage-dependent Ca\(^{2+}\) channels of the phenylalkylamine class, reduced the depolarization-induced activation of CaMKI by ~80%. Similar results were obtained with a Ca\(^{2+}\)-channel inhibitor of the dihydropyridine class, nifedipine (data not shown). The reduction of depolarization-induced CaMKI activation produced by D600 was accompanied by restoration of the ability of CaMKI to be phosphorylated by purified CaMKIK in vitro (Fig. 2B). Finally, chelation of extracellular Ca\(^{2+}\) with EGTA completely abolished CaMKI activation by depolarization (Fig. 3B). Taken together, these results indicate that the phosphorylation and activation of CaMKI induced by membrane depolarization requires the influx of extracellular Ca\(^{2+}\). In addition, the finding that EGTA, but not D600, lowered CaMKI activity in non-KCl-treated cells is consistent with a serum-stimulated Ca\(^{2+}\) influx via a pathway not involving voltage-dependent Ca\(^{2+}\) channels, which would result in measurable CaMKI phosphorylation and activation in non-agonist-treated cells.

DISCUSSION

In analogous fashion to observations leading to the elucidation of the MAPK signaling cascade (42–44), CaMKI was initially reported to require phosphorylation for its activity and to be subject to activation by a distinct protein “activator” (11, 12, 45). Subsequent purification and characterization of the CaMKI activator revealed it to be a protein kinase which directly phosphorylated and activated CaMKI, i.e. to be a CaMKI kinase (CaMKIK) (7, 17, 18, 21). With the identification of Thr-177 of CaMKI, first as the site of autophosphorylation (6) and then as the site for phosphorylation and activation by CaMKIK (7, 20, 21), further similarity with components of the MAPK cascade was hypothesized (6, 7) since the activating phosphorylation sites in both MAPKs and MAPK kinases (MAPKKs or MEKs) are situated at similar positions in protein kinase subdomain VIII (24–26) a region termed the “phosphorylation lip” or activation loop (22, 23). Unlike MAPKs and MAPKKs, however, CaMKI exhibits, in addition to regulation by phosphorylation, absolute dependence of its activity upon direct, allosteric activation (by Ca\(^{2+}\)-CaM) (7, 16), a dependence completely retained by the phosphorylated enzyme (12, 17). Thus, an alternative view to a signal-driven cascade model would be that Thr-177 is phosphorylated post-translationally, thereby priming CaMKI for rapid allosteric activation by Ca\(^{2+}\)-CaM.

We document here the existence of a CaMKI phosphorylation cascade in intact cells and show that initiation of this cascade is sensitive to stimuli-induced elevations of intracellular Ca\(^{2+}\) levels. Aspects of this cascade based on data presented here with PC12 cells are supported by a number of previous observations made in vitro using purified components. First, the rapid and biphasic nature of intracellular CaMKI phosphorylation (Fig. 1), combined with the ability of protein phosphatase 2A to reverse CaMKI activation in vitro (12), suggests the potential for a rapidly reversible phosphorylation-dephosphorylation cycle in situ as a means of responding to intracellular Ca\(^{2+}\) transients. Whether protein phosphatase 2A is the enzyme responsible for intracellular dephosphorylation of CaMKI remains to be established; however, we have observed that inclusion in the PC12 cell lysis buffer of the protein phospho-
elevation of intracellular Ca\textsuperscript{2+} phosphorylation and activation of CaMKIV, triggered by the Hanissian the existence of an intracellular CaMKIV phosphorylation cascade member of the CaMK group, CaMKIV, is phosphorylated and unpublished observations. not noted that although initiation of the cascade may absolutely 2A/1 inhibitor, okadaic acid, appears to be required to activation. 1–3) are consonant with the observation that in addition to its motion of Ca\textsuperscript{2+} significance. One area of particular interest is the nervous system, the CaMKI cascade may likewise be of broad biological significance. Both excitable and nonexcitable cells (49), coupled with the CaMKI/CaMKK pathway may also have functional and neuronal differentiation (56), offer the exciting possibility and, in addition, directly activates CaMKIK (7, 19, 21). These phosphorylation of the enzyme suitable for Thr-177 phosphorylation than was CaMKI from depolarized cells (1.3-fold activation). From nontreated cells was significantly more phosphorylated than was CaMKI from depolarized cells (1.3-fold activation). It has been observed that, in addition to CaMKI, another 271, 1080–10910 84, 875–887 20, 694–701 15. Sun, P., Lou, L., and Maurer, R. A. (1996) J. Biol. Chem. 270, 10501–10505 26. Gould, K. L., Moreno, S., Owen, D. J., Sazer, S. A., and Nurse, P. (1991) EMBO J. 10, 885–892 27. Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991) EMBO J. 10, 3297–3309 28. Gu, Y., Rosenblatt, J., and Morgan, D. O. (1991) EMBO J. 10, 3959–4005 29. Kato, Y.-I., Matsumoto, M., Strom, D. K., and Sherr, C. J. (1994) Mol. Cell. Biol. 14, 2713–2721 30. Sebert, A. M., Anderson, K. A., Huang, Q.-H., Goldstein, E. G., Means, A. R., and Edelman, A. M. (1995) J. Biol. Chem. 270, 17616–17621 31. Greene, L. A., Aletta, J. M., Rukenstein, A., and Green, S. H. (1987) Methods Enzymol. 147B, 207–216 32. Zhang, W., Dziak, R. M., and Aletta, J. M. (1995) J. Cell. Physiol. 162, 348–358 33. Czernik, A. J., Pang, D. T., and Greengard, P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7518–7522 34. Tokumitsu H., Brickey, D. A., Glod, J., Hidaka, H., Sikeja, J., and Soderling, T. R. (1984) J. Biol. Chem. 259, 28840–28847 35. Tokumitsu, H., Enslen, H., and Soderling, T. R. (1995) J. Biol. Chem. 270, 19320–19324 36. Lai, Y., Nairn, A. C., and Greengard, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4223–4227 37. Lou, L. L., Lloyd, S. J., and Schulman, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9407–9501 38. Miller, S. G., and Kennedy, M. B. (1988) Cell 44, 861–870 39. Schworer, C. M., Cobran, J. R., and Soderling, T. R. (1986) J. Biol. Chem. 261, 8581–8584 40. MacNicol, M., J. J. Dferson, A. B., and Schulman, H. (1990) J. Biol. Chem. 265, 18055–18058 41. Park, I.-K., and Soderling, T. R. (1995) J. Biol. Chem. 270, 30464–30469 42. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) Nature 343, 651–653 43. Aho, N. G., Seger, R., Bratlien, R. L., Diliz, C. D., Tonks, N. K., and Krebs, E. G. (1991) J. Biol. Chem. 266, 4220–4227 44. Gomez, N., and Cohen, P. (1991) Nature 353, 170–173 45. Mochizuki, H., Sugita, R., Ito, T., and Hidaka, H. (1993) Biochem. Biophys. Res. Commun. 197, 1356–1360 46. Okuno, S., Kitani, T., and Fujisawa, H. (1994) J. Biochem. (Tokyo) 116, 923–936 47. Tokumitsu, H., and Soderling, T. R. (1996) J. Biol. Chem. 271, 5617–5622 48. Hanissian, S. H., Frangakis, M. V., Bland, M. J., awahar, S., and Chatila, T. A. (1993) J. Biol. Chem. 268, 20055–20063 49. Clapham, D. E. (1995) Cell 80, 259–261 50. Ghosh, A., and Greenberg, M. E. (1993) Science 263, 239–247 51. Rusanesu, G., Qi, H.-Q., Thomas, S. M., Brugge, J. S., and Halegoua, S. (1995) Neuron 15, 1415–1429 52. Teng, K. K., and Greene, L. A. (1993) J. Neurosci. 13, 3124–3125 53. Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994) J. Biol. Chem. 269, 15520–15527 54. Matthews, R. P., Guthrie, C. R., Walle, M. L., Zhao, X., Means, A. R., and McKnight, G. S. (1994) Mol. Cell. Biol. 14, 6107–6116 55. Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995) Mol. Cell. Biol. 15, 3877–3884 56. Sokol, M., McMahon, T., and Messing, R. O. (1995) J. Neurosci. 15, 5966–5975