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Activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme

(signal transduction/phosphorylation/brain)

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ABSTRACT **The multifunctional Ca^{2+}/calmodulin-dependent protein kinase purified from rat brain cytosol undergoes an intramolecular self-phosphorylation or autophosphorylation. Autophosphorylation produces two strikingly different effects on kinase activity that are dependent on the level of ATP used in the reaction. At low but saturating levels of ATP (5 μM), autophosphorylation causes a 75% reduction in kinase activity, with the residual activity still retaining a dependence on Ca^{2+} and calmodulin. By contrast, at high but physiological levels of ATP (500 μM), the kinase is converted by autophosphorylation to a form that is autonomous of Ca^{2+} and calmodulin, with no accompanying reduction in activity. The extent of phosphate incorporation does not determine whether the kinase becomes inhibited or autonomous. Autophosphorylated kinase shows the functional change characteristic of the ATP concentration used during the reaction—inhibited at low ATP and autonomous at high ATP—even when compared at the same level of incorporated phosphate. ATP appears to regulate the site(s) phosphorylated during activation of the kinase and thereby modulates the dual effects of autophosphorylation. Events triggered by transient elevations of cellular Ca^{2+} may be potentiated and retained by generation of the Ca^{2+}/calmodulin-independent protein kinase activity.**

Intracellular Ca^{2+} rises in response to many neurotransmitters, hormones, growth factors, and other extracellular signals. This laboratory has reported on the purification and characterization of a soluble, multifunctional, Ca^{2+}/calmodulin-dependent protein kinase that may orchestrate some of the effects of these signals in brain and other tissues (1–4). An identical enzyme has been described by others in brain (5–9), and a related enzyme has been described in nonneuronal (3, 4, 10–13) and nonmammalian tissues (3, 14–16). We refer to this enzyme as the multifunctional Ca^{2+}/calmodulin-dependent protein kinase because it appears to have a broad, yet limited, substrate specificity that clearly distinguishes it from phosphorylase kinase and myosin light chain kinase, two dedicated Ca^{2+}/calmodulin-dependent protein kinases with very strict substrate specificity (3, 17). The same kinase has also been referred to as the "Ca^{2+}/calmodulin-dependent protein kinase II or type II" (6, 9, 18). This kinase was first identified in membrane fractions from numerous tissues (19, 20) but is abundant in cytosol as well (1, 5–9). The particulate form is also associated with the "cytoskeleton" (21, 22), the nucleus (23, 24), and a neuronal specialization termed the postsynaptic density (25–27). The kinase phosphorylates synapsin I (28) and tyrosine hydroxylase (4) *in situ*. Many other proteins, including microtubule-associated protein 2 (MAP-2), τ proteins, tryptophan 5'-monooxygenase, ribosomal protein S6, vimentin, and myosin light chains, are good substrates *in vitro* and are candidates for physiological substrates (3, 6, 13). Therefore, the Ca^{2+}/calmodulin-dependent protein kinase may be involved in the regulation and coordination of numerous cellular processes. As such, it would not be surprising to find that the enzyme is itself under regulatory control.

A common mechanism for modulating the activity of protein kinases is autophosphorylation (29–32). The soluble Ca^{2+}/calmodulin-dependent protein kinase that we purified from rat forebrain autophosphorylates when stimulated by Ca^{2+} and calmodulin (2, 7, 8, 33). The kinase consists of a 10-subunit holoenzyme of 580-kDa composed of structurally related subunits of 51 kDa and 60 kDa in a 4:1 ratio (1, 2). Both subunits become phosphorylated, incorporating from 1 to 3 mol of *P* per subunit (2, 8). We have shown that the autophosphorylation is an intramolecular reaction displaying saturation kinetics (33).

We have found that autophosphorylation of the rat brain enzyme leads to a significant inhibition of kinase activity (33). Similar findings for the brain and muscle enzyme have been reported independently (34). By contrast, others find no change in activity of the muscle kinase after autophosphorylation (11). Both inhibition and activation have been reported for the cytoskeletal (35) and postsynaptic density (36) forms of the rat brain enzyme. These resulted from changes in the calmodulin-binding properties of the kinase. In cytoskeletal preparations from *Aplysia* neurons, autophosphorylation of the molluscan form of this kinase leads to its release from the cytoskeleton and conversion to a Ca^{2+}/calmodulin-independent form (22). A combination of 70% inhibition and partial Ca^{2+}/calmodulin independence has been found recently with the rat brain enzyme (37).

We present evidence for dual modulation of the Ca^{2+}/calmodulin-dependent protein kinase from rat brain. At ATP concentrations approximating the K_m of the enzyme, autophosphorylation results in an inhibition of the kinase as reported (33). At higher ATP concentrations nearing physiological levels, autophosphorylation, while causing no inhibition, produces an autonomous kinase with full activity in the absence of both Ca^{2+} and calmodulin.

EXPERIMENTAL PROCEDURES

Material. *Staphylococcus aureus* V8 protease was obtained from Miles. $NaDodSO_4$ electrophoresis reagents (other than acrylamide) were from Bio-Rad. [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. Calmidazolium was purchased from Boehringer Mannheim. All other chemicals were obtained from Sigma. Calmodulin was prepared as described (20). Ca^{2+}/calmodulin-dependent protein kinase was purified from rat brain cytosol as de-

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Abbreviation: MAP-2, microtubule-associated protein 2.

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scribed (1) and has a specific activity of 0.3 μmol/min per mg in the MAP-2 kinase assay.

Purification of MAP-2. Microtubules were prepared by two cycles of assembly and disassembly from bovine brain cortical grey matter (38). MAP-2 was obtained in purified form after resolution on a Bio-Gel A-1.5m column.

Preparation of Autophosphorylated Kinase. Purified kinase was autophosphorylated by a modification of published procedures (33). The standard reaction mixture contained 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 500 μM $CaCl_2$, 5 μg of calmodulin and 1 mg of bovine serum albumin per ml, and ATP or [γ-^{32}P]ATP (1 Ci/mmol) as indicated. For determination of ^{32}P incorporation, reactions were initiated by addition of 325 ng of kinase in a final volume of 100 μl and maintained at 30°C for the indicated times. Kinase subunits were separated by $NaDodSO_4$ gel electrophoresis as described (39). Radioactive bands were located by autoradiography and excised from the gel; radioactivity was quantified by Cerenkov radiation by using a Beckman LS-3801 Liquid Scintillation System at 60% efficiency.

For experiments designed to assess the effect of autophosphorylation, purified kinase (975 ng) was added to a standard reaction mixture in a final volume of 30 μl containing the indicated amounts of unlabeled ATP. This "preincubation" was maintained at 30°C for the indicated times, and autophosphorylation was terminated by rapidly chilling the reaction tubes on ice and adding an equal volume of 2 mM EGTA. Reaction mixtures were supplemented with Ca^{2+}, calmodulin, and/or ATP to make their concentration in each sample equivalent, and MAP-2 kinase activity was performed immediately.

MAP-2 Kinase Assay. Activity of autophosphorylated kinase was determined by MAP-2 phosphorylation. The standard assay of initial velocity was performed as described (1) by using 0.5–1.0 μg of MAP-2 and 20 μM [γ-^{32}P]ATP (1 Ci/mmol) and was analyzed by counting Cerenkov radiation of excised gel bands as described above. The effect of the small amount of Ca^{2+} (12.5 μM) and calmodulin (0.125 μg/ml) carried over from the preincubation mixture was eliminated by addition of 200 μM EGTA in MAP-2 kinase assay designed to determine the "unstimulated" activity.

Dephosphorylation of Autophosphorylated Kinase. Autophosphorylated kinase was prepared as described above using 500 μM [γ-^{32}P]ATP. After 30 sec at 30°C, the reaction was terminated by addition of an equal volume of ice-cold 20 mM EDTA. The mixture was divided in two and incubated with either 4.5 units of purified catalytic subunit of protein phosphatase 2A or an equal volume of buffer (control), and samples were incubated at 30°C for 2 min. Dephosphorylation was determined by $NaDodSO_4$ gels, and autoradiography was as described above. Identical reactions containing unlabeled ATP were used to test for reversal of autonomy after dephosphorylation. MAP-2 phosphorylation under stimulated and unstimulated conditions was measured as described above. The phosphatase, purified to homogeneity (40), was the gift of Thomas S. Ingebritsen (University of Colorado Health Science Center).

RESULTS AND DISCUSSION

Activity of the Ca^{2+}/calmodulin-dependent protein kinase is tightly regulated by Ca^{2+} and calmodulin. Only negligible self-phosphorylation or phosphorylation of MAP-2 and other substrates is detected in the absence of either Ca^{2+} or calmodulin (1). While studying the autophosphorylation, we found a paradoxical effect of ATP, present during autophosphorylation, on the activity of the kinase. This dramatic dual modulation by ATP is shown in Fig. 1. We preincubated the kinase without substrate under standard conditions in either the absence of Ca^{2+} and calmodulin with 500 μM ATP (bars C; control) or the presence of Ca^{2+} and calmodulin with either 5 μM ATP (bars L; low ATP) or with 500 μM ATP (bars H; high ATP) as described. Functional consequences of autophosphorylation occurring during this preincubation were subsequently determined by diluting the preincubation mixture and assaying the kinase with MAP-2 as substrate. Kinase activity in the presence of Ca^{2+}/calmodulin (stimulated activity) is shown in Fig. 1 *Left*. The activity of control kinase, which does not become phosphorylated during the preincubation, is defined as 100%. As previously shown (33), autophosphorylation at low ATP (approximating the K_m for phosphorylation) results in an inhibition of kinase activity by 75% (Fig. 1 *Left*, bar L). Surprisingly, when we increased ATP to 500 μM or above, we saw no inhibition. There is, in fact, a slight increase in maximal activity because the kinase autophosphorylated at high ATP is more stable over the preincubation period at 30°C (data not shown) than is control kinase, to which its activity is normalized. Kinase activity in the absence of Ca^{2+}/calmodulin (unstimulated activity) is shown in Fig. 1 *Right*. Control kinase and kinase autophosphorylated with low ATP are inactive when not stimulated. Autophosphorylation with high ATP, however, produces a Ca^{2+}/calmodulin-independent kinase. Thus, autophosphorylation at high ATP (500 μM) converts the kinase, normally stringently regulated by Ca^{2+} and calmodulin, into an autonomous kinase capable of maximally phosphorylating exogenous substrates, even in the absence of Ca^{2+} and calmodulin (Fig. 1 *Right*, compare bar H with bar C or L). Phosphorylation of other substrates, including glycogen synthase, τ proteins, and synapsin I, also becomes autonomous (data not shown). Kinetic analysis of the phosphorylation at high ATP, performed as described at low ATP (33),

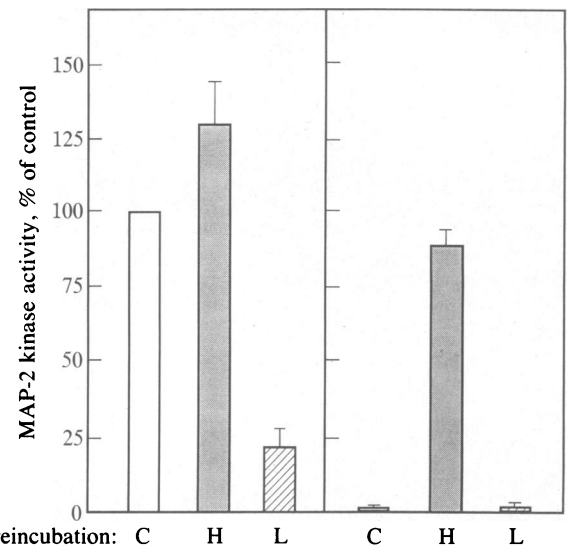

FIG. 1. Dual modulation of the Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation. Autophosphorylated kinase was prepared by incubating purified kinase at 5 μM (bars L) or 500 μM (bars H) ATP for 4 min as described. Control kinase (bars C) was processed under identical conditions to those of bars H except that Ca^{2+} and calmodulin were omitted. The effect of the preincubation was determined by immediately assaying kinase activity at a 1:40 dilution from the preincubation and using MAP-2 as substrate. The levels of ATP, Ca^{2+}, EGTA, and calmodulin were adjusted so that samples C, L, and H carried over the same amounts of these components to the second step of analysis. (*Left*) Ca^{2+} and calmodulin were included in the assay of MAP-2 phosphorylation (stimulated activity). (*Right*) Ca^{2+} and calmodulin were omitted in the assay of MAP-2 phosphorylation (unstimulated activity). In addition, 0.2 mM EGTA was included to chelate any Ca^{2+} carried over from the preincubation. All values were normalized to that of control kinase assayed in the presence of added Ca^{2+}/calmodulin (control activity in *Left*).

indicates that it is an intramolecular reaction (unpublished data). Therefore, it represents a self-phosphorylation or autophosphorylation by the kinase rather than phosphorylation by another Ca^{2+}/calmodulin kinase with a high K_m for ATP.

The Ca^{2+}/calmodulin-independent activity is not due to carry-over of Ca^{2+} and calmodulin from the autophosphorylation reaction because the concentrations of Ca^{2+}, calmodulin, and ATP were adjusted after the preincubation to assure equal concentrations in each of the three samples. Furthermore, we show in Table 1 that the unstimulated activity is resistant to inhibition by EGTA and by calmidazolium and trifluoperazine, two potent calmodulin antagonists (41, 42). They did block the residual Ca^{2+}/calmodulin dependence of the activity (data not shown). Under conditions used in Fig. 1 and Table 1, the autophosphorylated kinase assayed in the absence of Ca^{2+} and calmodulin had 90% of the activity of the stimulated control.

We show in Table 1 that the switch to a Ca^{2+}/calmodulin-independent activity is a consequence of phosphorylation rather than a direct stimulatory effect of ATP or an indirect effect of proteolysis. The switch requires Ca^{2+}, calmodulin, and ATP, the same components found essential for autophosphorylation. Addition of calmidazolium, trifluoperazine, or excess EGTA or omission of ATP completely prevents production of an autonomous kinase (Table 1). Finally, attempts to block autonomy, produced by autophosphorylation, with various protease inhibitors (Table 1) were unsuccessful.

In preliminary studies, we find that both phosphorylation and autonomy can be reversed by protein phosphatase 2A. Autonomous kinase was generated by incubation with Ca^{2+}/calmodulin and 500 μM ATP. Phosphorylation was terminated by addition of EDTA, and dephosphorylation was initiated with protein phosphatase 2A (40) as described. Autonomous MAP-2 kinase activity was reversed by 80.9% ± 5.9%. The neuronal form of this kinase is a major phosphatase activity in rat forebrain cytosol and has been shown to dephosphorylate the Ca^{2+}/calmodulin-dependent protein kinase in synaptic junctions (43).

Several lines of evidence argue against trapping of calmodulin by the kinase as a mechanism for generation of the autonomous activity. We measured calmodulin binding using the ^{125}I-labeled calmodulin overlay method (44) and found that the autonomous kinase has 87% of control level of binding and that the binding is completely dependent on the presence of Ca^{2+}. The autonomous kinase adsorbs to a calmodulin-Sepharose affinity column in the presence of Ca^{2+} and is eluted with EGTA (data not shown). Finally, resolution of the kinase from other components of the reaction mixture by elution on Sepharose 6B with excess EGTA and assay for activity shows the kinase to be 80% autonomous.

Table 1. Generation and characteristics of the autonomous kinase

Autophosphorylation reaction	Kinase assay (unstimulated)	Autonomous activity, %
Complete	Complete	(100.0)
Complete	+ 1 μM CDZ	92.0 ± 6.3
Complete	+ 20 μM TFP	99.0 ± 14.5
Complete	+ 1 mM EGTA	101.5 ± 2.9
− ATP	Complete	1.0 ± 0.8
+ 1 μM CDZ	Complete	2.9 ± 1.6
+ 20 μM TFP	Complete	4.0 ± 2.0
+ 10 mM EGTA	Complete	1.7 ± 1.5
+ 100 μg of leupeptin and aprotinin per ml	Complete	91.5 ± 0.3

A preparation of autonomous kinase was generated from purified kinase preincubated at 500 μM ATP as described. The MAP-2 activity of the kinase (assayed as in the legend to Fig. 1 *Right*) is 90% of stimulated control activity and is normalized above to 100%. Variations in either preincubation or MAP-2 phosphorylation are indicated. Each value represents the average of at least three determinations ± SEM. CDZ, calmidazolium; TFP, trifluoperazine.

We next examined the possibility that the dual effect of autophosphorylation at high and low ATP reflect different extents of phosphate incorporation. The K_m of ATP for autophosphorylation in the experiments above is 1.6×10^{-6} M. The two concentrations of ATP used in Fig. 1, 5 and 500 μM, are more than 3- and 300-fold higher than the K_m, respectively. Thus, there should be only a small difference in phosphate incorporated between autophosphorylation carried out at low and high ATP. We chose 1 μM and 500 μM ATP to further exaggerate the difference and prepared kinase samples autophosphorylated to various extents at each concentration of ATP by varying the time of preincubation. The time course of phosphate incorporation into the kinase is shown in Fig. 2 *Top*. As anticipated, phosphorylation at 500 μM ATP is several times faster than phosphorylation at 1 μM ATP.

We tested the possibility that inhibition and autonomy simply reflect different extents of phosphate incorporation. The effect of increasing phosphorylation on stimulated activity is shown in Fig. 2 *Middle*. In fact, autophosphorylation at high ATP causes no inhibition even at the lowest levels of phosphate incorporation. Autophosphorylation at low ATP quickly inhibits the activity and this is not reversed even when 4 mol of phosphate have been incorporated. Fig. 2 *Bottom* shows the effect of phosphorylation on the unstimulated activity. At high ATP there is a time-dependent increase in autonomous activity consistent with the Ca^{2+}/calmodulin-independent state being a consequence of phosphate incorporation rather than the mere presence of high ATP. At low ATP, the maximally inhibited enzyme is still dependent on Ca^{2+}/calmodulin and thus shows little unstimulated activity. Paradoxically, the extent of phosphorylation does not predict the direction or nature of the functional effect of autophosphorylation. For example, the kinase preincubated with low ATP incorporates 3 mol of *P* per holoenzyme by 2 min, with a resulting 50% inhibition. The kinase preincubated with high ATP incorporates 3 mol of *P* by 15 sec, at which point it is 50% autonomous and still has maximal activity when assayed with Ca^{2+}/calmodulin.

The explanation for the paradox is that it is not the degree of phosphorylation *per se* but the site(s) of phosphorylation that may determine which mode of regulation occurs. We and others (7, 8, 33) have shown that the kinase shows anomalous behavior on $NaDodSO_4$ gels after autophosphorylation. The presence of phosphate at some critical site(s) on these polypeptides, perhaps by interfering with $NaDodSO_4$ binding, results in a distinct shift of the 51-kDa subunit to a band at 53 kDa. Fig. 3 *Left* shows that this shift is only seen with autophosphorylation at low ATP. Phosphorylation at high ATP, under conditions that produce an autonomous kinase, apparently does not phosphorylate the same critical site(s) and, therefore, causes no shift to higher molecular weight. This suggests that investigators reporting such a shift (7, 8, 33) were not phosphorylating the kinase under conditions leading to an autonomous activity.

Partial proteolysis also reveals distinct differences in phosphopeptides between inhibited and autonomous enzyme (Fig. 3 *Right*). Whereas most of the larger phosphopeptides are common to both preparations, several distinct bands in the 10- to 20-kDa region can be clearly discerned. Similar differences were consistently seen at various degrees of proteolysis. Extensive analysis using tryptic peptide mapping suggests that extra phosphopeptides are found in kinase autophosphorylated at low ATP (unpublished data). These extra peptides are suppressed at high ATP as suggested by

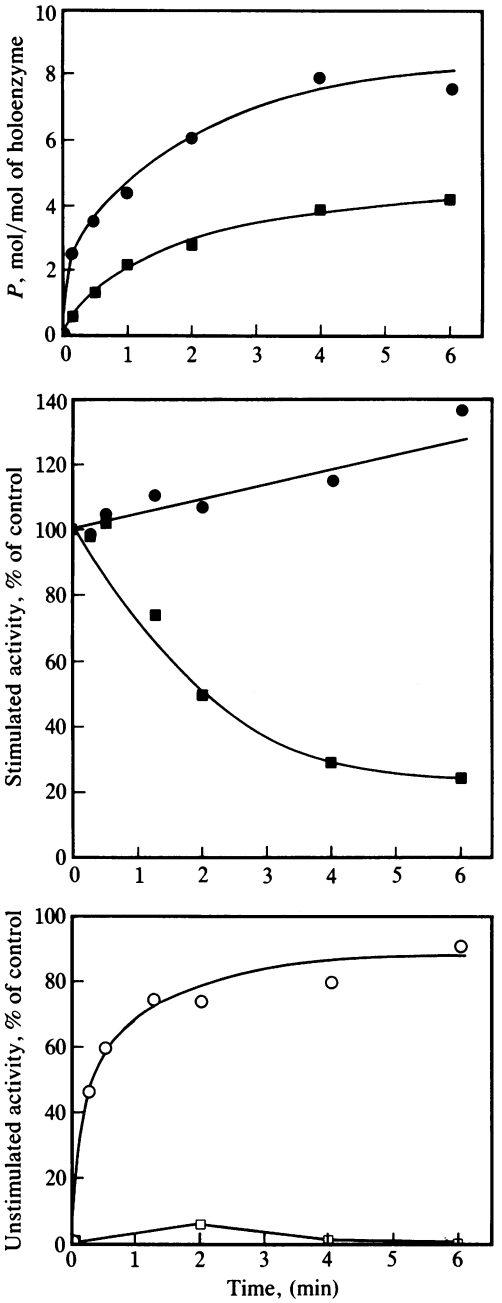

FIG. 2. The effect on kinase activity of phosphate incorporated during autophosphorylation with low and high ATP. (*Top*) Kinase was autophosphorylated under standard conditions at 1 μM (■) or 500 μM (●) [γ-^{32}P]ATP. At the indicated times, aliquots were taken, and ^{32}P incorporated into the holoenzyme was measured as described. Because of differential dye binding, the concentration of kinase is 3-fold higher in the Bradford assay using bovine gamma globulin than in the Lowry assay using bovine serum albumin as standard. Phosphate incorporation is therefore 3-fold higher than shown when protein is measured by the Lowry assay. (*Middle*) Kinase was autophosphorylated as described above using nonradioactive ATP. Control kinase (not autophosphorylated) was treated by the same procedure except that ATP was omitted in the preincubation. After the times indicated, MAP-2 phosphorylated under stimulated conditions (with Ca^{2+}/calmodulin) was determined by using a 30-sec incubation for samples autophosphorylated at 1 μM (■) or 500 μM (●) ATP. All values are expressed as percentage of control, with the stimulated control being 100%. (*Bottom*) Same as *Middle* except that MAP-2 kinase activity was assayed under unstimulated conditions (no added Ca^{2+}/calmodulin) for samples autophosphorylated at 1 μM (□) or 500 μM (○) ATP.

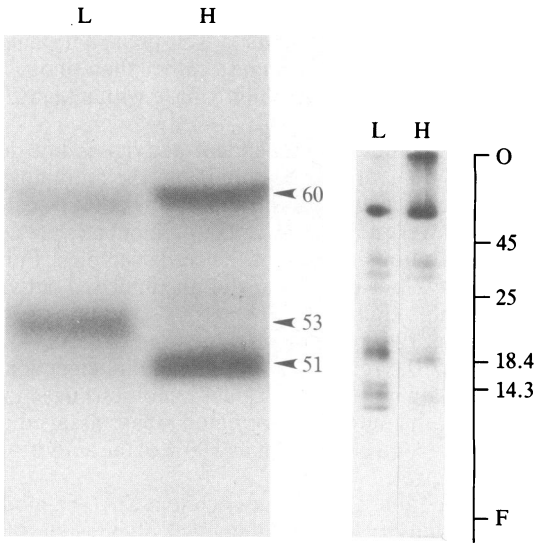

FIG. 3. Comparison of kinase autophosphorylated with low and high ATP by mobility on $NaDodSO_4$ gels and by partial proteolysis. Kinase was phosphorylated to the same extent of ^{32}P incorporation under standard conditions with 1 μM (lanes L) and 500 μM (lanes H) [γ-^{32}P]ATP. The reaction was incubated for 1 min at high ATP and 4 min at low ATP. (*Left*) Autoradiograph of kinase subunits resolved on $NaDodSO_4$/8% polyacrylamide gels (45). Only the 50- to 60-kDa region is shown. (*Right*) Partial proteolysis of the smaller kinase subunit with 10 μg of *Staphylococcus aureus* V8 protease per lane was performed as described (45). Similar results were obtained at various concentrations of protease. Molecular weights are indicated in kDa. O, Origin; F, dye front.

the protease V8 digests (Fig. 3 *Right*) and confirmed by tryptic mapping. Therefore, high ATP may block phosphorylation that would lead to inhibition and thus permits the autonomy to be expressed. At low ATP, phosphorylation of the extra sites leads to the dominant effect, the inhibition. The sites of phosphorylation and the calmodulin-binding domains of the kinase overlap to some extent (36). Depending on their precise location, the phosphates either may inhibit the interaction with calmodulin, leading to an inhibition, or may mimic the change elicited by interaction with calmodulin, leading to autonomy. ATP may bind at an allosteric site and modulate autophosphorylation, thereby controlling the ratio of inhibited and autonomous forms of the kinase without appreciably affecting total phosphate incorporation.

The Ca^{2+}/calmodulin-dependent protein kinase appears to be a multifunctional enzyme regulating numerous processes (3, 9, 46). Regulation by autophosphorylation may be an important mechanism for modulating its activity. The physiological level of ATP in brain tissue is 2.8 mM (47). Therefore, it would seem that generation of the autonomous state, which occurs at physiological levels of ATP, rather than the inhibition, which occurs far below physiological levels, is the most likely consequence of autophosphorylation *in vivo*. However, other conditions present in cells may modify the ATP requirement in a way not reproduced *in vitro* and permit both modes of regulation to occur. It is also possible that phosphorylation at low ATP has revealed a site not normally autophosphorylated *in vivo* but phosphorylated by some other kinase that functions to regulate (inhibit) the Ca^{2+}/calmodulin-dependent protein kinase.

Generation of an autonomous Ca^{2+}/calmodulin-dependent protein kinase has been shown to occur in cytoskeletal preparations from *Aplysia* neurons (22). Under phosphorylating conditions, which include Ca^{2+}/calmodulin and ATP (1 mM), the molluscan counterpart of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase was released from a cytoskeleton-bound state and became autonomous of

regulation by Ca^{2+}/calmodulin. We believe that the autonomy seen in the *Aplysia* system is the same phenomenon being described here for the purified cytosolic enzyme from rat brain. Whether release from the cytoskeleton also accompanies autophosphorylation of the cytoskeletal form of the mammalian enzyme needs to be investigated. Our finding of dual modulation by ATP explains why the phenomenon of autonomy has not been described previously and why it was found in *Aplysia*. Most investigators, ourselves included, have used the customary low concentrations of ATP during autophosphorylation to optimize the specific activity of [γ-^{32}P]ATP. Depending on the ATP level used, various degrees of inhibition and autonomy can be obtained.

Processing of information in neuronal and nonneuronal systems involves responses to both the frequency and the magnitude of stimulation. It is tempting to speculate that activation of the neuronal Ca^{2+}/calmodulin-dependent protein kinase *in vivo* is accompanied by some autophosphorylation and that the history of synaptic activity involving rapid fluctuations in Ca^{2+} levels may be retained by the kinase for some period of time. Generation of an autonomous state would tend to both potentiate and prolong the activity of the kinase and its physiological effects because Ca^{2+} elevation is transient. The corollary of this model is that a protein phosphatase(s), perhaps protein phosphatase 2A or 1, which are known to dephosphorylate the kinase (43), would have an important role in terminating this autonomous state. It is also possible that persistent synaptic activity, perhaps coupled to inhibition of the phosphatase, may convert a sufficient amount of the kinase to the autonomous state to overcome reversal by the phosphatase. Theoretical models for such summation of synaptic activity have recently been presented (48, 49) and provide a framework for testing this hypothesis.

After this manuscript was submitted, two reports demonstrating the conversion of the kinase to a Ca^{2+}/calmodulin-independent form were published (50, 51). In one of the studies (50), autonomy without inhibition was generated at both low and high ATP. The apparent discrepancy with our finding is a result of differing reaction conditions. We have also found that at 0°C, the temperature in the other study, no inhibition is seen even at low ATP. However, this is because, at the lower temperature, the kinase reaction is not only slowed—the intended result—but also is modified so that different sites are autophosphorylated than at the standard temperature. At 0°C and low ATP, there is no shift in mobility of the kinase subunits on $NaDodSO_4$ gels comparable to what is seen at 30°C and high ATP. Thus, lowering the temperature has the functional consequence of raising the ATP.

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