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Correlation of Activation of Ca^{2+}/Calmodulin-Dependent Protein Kinase II with Catecholamine Secretion and Tyrosine Hydroxylase Activation in Cultured Bovine Adrenal Medullary Cells

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SUMMARY

We have investigated the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in cultured bovine adrenal medullary cells. The activation was assayed as an increase in the Ca^{2+}-independent (autonomous) activity of CaM kinase II, using the synthetic substrate Syntide-2. Incubation of cells with acetylcholine increased the Ca^{2+}-independent activity in a time (20 sec to 5.0 min)- and concentration (10–300 μM)-dependent manner. These curves were closely correlated with those of catecholamine secretion and tyrosine hydroxylase activation. Removal of extracellular Ca^{2+} completely abolished the stimulatory effects of acetylcholine on the Ca^{2+}-independent activity, as well as on catecholamine secretion and activation of tyrosine hydroxylase. Nicotine but not muscarine increased the Ca^{2+}-independent activity as potently as did acetylcholine, and hexamethonium but not atropine completely blocked the acetylcholine-induced increase. In ^{32}P-labeled cells, acetylcholine stimulated the phosphorylation of a 50-kDa protein that was immunoprecipitated with an anti-brain CaM kinase II antibody. These results suggest that acetylcholine stimulates CaM kinase II activity through nicotinic acetylcholine receptor-mediated influx of Ca^{2+} and that the activation of CaM kinase II is closely related to catecholamine secretion and tyrosine hydroxylase activation in cultured adrenal medullary cells.

CaM kinase II is widely distributed throughout many tissues and has a broad substrate specificity, like cAMP-dependent protein kinase or PKC (1, 2). This kinase is, therefore, considered to be involved in the regulation of numerous Ca^{2+}-mediated cellular processes. CaM kinase II exists as a heteropolymer that is composed of 10–12 subunits, including α, β, β', γ, and δ subunits, and the molecular mass of the CaM kinase II holoenzyme is 300–600 kDa (1, 2). Previous *in vitro* studies have revealed a unique activation property of CaM kinase II, that is, autophosphorylation of the kinase converts it from the Ca^{2+}-dependent form to the Ca^{2+}-independent (autonomous) form. In the absence of Ca^{2+}/calmodulin, CaM kinase II is inactive due to the interaction of the catalytic domain (residues 1–260) with the regulatory domain (residues 281–309), which contains the autoinhibitory domain and the Ca^{2+}/calmodulin binding site (1, 2). In the presence of Ca^{2+}/calmodulin, the binding of Ca^{2+}/calmodulin to the kinase (3, 4) and the subsequent autophosphorylation of Thr-286/287 (α/β subunits, respectively) (5–7) can remove the inhibitory potency of the regulatory domain. These make the kinase constitutively active and maintain the activation of CaM kinase II even when Ca^{2+} is removed. This is referred to as the Ca^{2+}-independent (autonomous) activity of the kinase.

In adrenal medullary cells, stimulation of acetylcholine receptors physiologically causes the synthesis (8) and secretion (9) of catecholamines, in a Ca^{2+}-dependent manner. An increase in catecholamine synthesis is associated with the activation of tyrosine hydroxylase, the rate-limiting enzyme (10). Accumulating evidence has indicated that protein phosphorylation is associated with stimulus-secretion coupling and plays a role in the regulation of catecholamine synthesis (8, 11–14). CaM kinase II is noted as a possible candidate that mediates the effect of Ca^{2+}. Indeed, the phosphopeptide map of tyrosine hydroxylase stimulated by carbachol or depolarization *in situ* is similar to that of the enzyme phosphorylated by CaM kinase

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ABBREVIATIONS: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PKC, protein kinase C.

II *in vitro* (15–18). Recently, we have demonstrated that an isoenzyme of CaM kinase II exists in cultured bovine adrenal medullary cells (19). The kinase phosphorylates tyrosine hydroxylase, several proteins of chromaffin granule membranes, and a novel endogenous substrate (70 kDa) *in vitro* (19). However, the activation of CaM kinase II has not been investigated in association with the functional responses in intact adrenal medullary cells.

On the basis of the unique regulatory properties of the CaM kinase II activity described above, an assay method for kinase activation at the cell level has been established. In intact cells the autophosphorylation of CaM kinase II is well correlated with the generation of Ca^{2+}-independent CaM kinase II activity (13, 20). The assay of Ca^{2+}-independent activity is simple and sensitive for selectively measuring CaM kinase II activity *in situ* (13, 20, 21). Therefore, the Ca^{2+}-independent activity has been used as a useful index of CaM kinase II activation in various intact cells (13, 20–26). In the present study, we have examined the relationship between activation of CaM kinase II and catecholamine secretion or tyrosine hydroxylase activation in acetylcholine-stimulated adrenal medullary cells.

Experimental Procedures

Materials. The following chemicals and reagents were obtained from the indicated sources: Eagle's minimum essential medium, Nissui Seiyaku; collagenase, Nitta Zerachin; tetrodotoxin, Sankyo; hexamethonium, Tokyo Kasei; acetylcholine, nicotine, atropine, histamine, and calf serum, Nacalai Tesque; bradykinin, Peptide Institute; ionomycin, Calbiochem; veratridine, *dl*-muscarine, soybean trypsin inhibitor, cytosine arabinoside, and amphotericin B, Sigma; aminobenzylpenicillin and streptomycin, Meiji Seika; Protein A-Sepharose CL-4B, Pharmacia LKB Biotechnology; molecular weight markers for SDS-PAGE, Bio-Rad; [^{32}P]P_i (500 mCi/ml), ICN Biochemicals; [γ-^{32}P]ATP (3000 Ci/mmol), Amersham International; L-[1-^{14}C]tyrosine (53.4 mCi/mmol), New England Nuclear; catalase, Boehringer Mannheim; calmodulin from bovine brain and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, Calbiochem-Behring; CaM kinase II substrate peptide Syntide-2 and synthetic peptide inhibitors of cAMP-dependent protein kinase (PKI-tide) and PKC (PKC_{19-36}), Bachem Feinchemkalien AG; P-cellulose papers, Whatman. The polyclonal antibody (IgG fraction) against rat brain CaM kinase II was prepared as described previously (22, 27). Other chemicals used were of analytical grade from Nacalai Tesque.

Cell culture. Fresh bovine adrenal glands were used for all experiments. Isolated adrenal medullary cells were obtained by collagenase digestion of slices of adrenal medulla, as reported previously (28). To remove nonchromaffin cells such as fibroblasts or epithelial cells, the differential plating procedure was used (29). In brief, the isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum, 3.0 μM cytosine arabinoside, 60 μg/ml aminobenzylpenicillin, 100 μg/ml streptomycin, and 0.3 μg/ml amphotericin B (30) and were plated on dishes for 3 hr at 37° in 5% CO_2/95% air. The nonattached cells (adrenal medullary cells) were decanted away from the attached cells and maintained in monolayer culture at a density of 4×10^6 cells/dish (Falcon 35-mm dishes). The cells were used for experiments within 2 or 3 days of culture. The purity of the cultured cells was confirmed by the Grimelius method (31), and the final cell preparation contained at least 80–90% chromaffin cells.

CaM kinase II activity. Oxygenated Krebs-Ringer phosphate buffer was used in the experiments for determination of CaM kinase II activity, catecholamine secretion, and tyrosine hydroxylase activity in the cells. It was composed of 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, and 10 mM glucose, adjusted to pH 7.4. The cells were incubated with the test agents in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4 at 37°, for the indicated periods.

For the termination of cell stimulation, the incubation medium was quickly aspirated and the cells were frozen on dry ice. The frozen cells were scraped and homogenized in 160 μl of solubilization medium, which contained 50 mM HEPES buffer, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 5 mM EDTA, 15 mM $Na_4P_2O_4$, 100 mM β-glycerophosphate, 25 mM NaF, 0.43 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 75 μM pepstatin. After centrifugation at 15,000 × *g* for 5 min, aliquots of the supernatant were assayed for CaM kinase II activity (20). The standard CaM kinase II assay mixture contained 50 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (3000–5000 cpm/pmol), 1 mg/ml bovine serum albumin, 40 μM Syntide-2 (CaM kinase II substrate peptide), 0.1 mM PKI-tide, and 1 μM PKC_{19-36}, with 1 mM EGTA (Ca^{2+}-independent activity) or with 1 mM $CaCl_2$ and 2 μM calmodulin (total activity), in a volume of 20 μl. The reaction was initiated by the addition of 5 μl of the supernatant and was carried out at 30° for 2 min. After incubation, 20 μl of an aliquot of each sample were spotted on a P-cellulose paper square and processed as described by Roskoski (32). Because the total CaM kinase II activity determined with Ca^{2+}/calmodulin did not change in any of the experiments, the ratio of the Ca^{2+}-independent activity to the total activity was used to represent the marker of activation of the enzyme.

The assay for the Ca^{2+}-independent activity using a good substrate for CaM kinase II (Syntide-2) (33) and specific inhibitors of cAMP-dependent protein kinase (PKI-tide) and PKC (PKC_{19-36}) (34) has been shown to be selective for measuring CaM kinase II activity (13, 20, 21). The Ca^{2+}-independent and total CaM kinase II activities determined here were blocked by a synthetic inhibitory peptide of CaM kinase II (amino acids 281–309), which inhibited purified brain CaM kinase II. Syntide-2, which was synthesized as a highly homologous peptide for the site of phosphorylation of glycogen synthase by CaM kinase II, served as only a poor substrate for PKC (33). The PKC inhibitor PKC_{19-36} (1 μM), which almost completely inhibits the activity of purified brain PKC, had little effect on either the Ca^{2+}-independent or total CaM kinase II activity determined in the present assay system.

Catecholamine secretion. After the reaction with the test agents, the incubation medium (1 ml) was immediately transferred to a test tube containing 4 ml of ice-cold 0.5 M perchloric acid. The catecholamines secreted into the medium were adsorbed into aluminum hydroxide and estimated by the ethylenediamine condensation method, using a fluorescence spectrophotometer (Hitachi 650–10S) with an excitation wavelength of 420 nm and an emission wavelength of 540 nm (35). At these wavelengths, epinephrine and norepinephrine showed the same fluorescence intensities. The secreted catecholamines were expressed as the percentage of total catecholamines (epinephrine plus norepinephrine) in the cells.

Tyrosine hydroxylase activity. After cell stimulation, the cells were harvested with 200 μl of buffer containing 30 mM potassium phosphate, pH 6.8, 50 mM NaF, and 1 mM EDTA. The cell suspension was homogenized and centrifuged at 20,000 × *g* for 10 min, and the resultant supernatant was applied to a Sephadex G-25 column (0.9 × 3.1 cm). The tyrosine hydroxylase activity in the effluent was measured by a modified decarboxylase-coupled assay (10, 11). Because the reactions were observed to be linear up to 60 μl of enzyme volume and for 20 min, the assay was performed with 55 μl of enzyme solution for 10 min. The incubation mixture (100 μl) contained 100 mM potassium phosphate buffer, pH 6.8, 5 mM ascorbate, 5 mM EDTA, 6500 units of catalase, 0.25 mM 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 0.1 mM L-[1-^{14}C]tyrosine (0.1 μCi), and the enzyme. The specific activity of tyrosine hydroxylase was expressed in nanomoles of $^{14}CO_2$ formed/minute/milligram of protein. The protein was determined according to the method of Lowry *et al.* (36), using bovine serum albumin as a standard.

Labeling of cells with [^{32}P]P_i and immunoprecipitation with the anti-CaM kinase II antibody. The cultured bovine adrenal medullary cells were washed four times with 1 ml of oxygenated Krebs-

Ringer-HEPES buffer, which contained 125 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 25 mM HEPES, and 10 mM glucose, pH 7.4. The cells were labeled with [^{32}P]P_i (0.5 mCi/ml) at 37° for 1 hr in 1.0 ml of Krebs-Ringer-HEPES buffer, pH 7.4. The cells were then washed and stimulated, with or without 0.3 mM acetylcholine, at 37° for 3 min. After stimulation, the medium was quickly aspirated and the cells were frozen on dry ice. The frozen cells were scraped from the dishes and homogenized in 160 μl of solubilization solution consisting of 50 mM HEPES, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 0.43 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 mg/ml aprotinin. After centrifugation at 15,000 × *g* for 15 min to remove insoluble materials, the supernatants were incubated at 4° overnight with affinity-purified rat brain anti-CaM kinase II antibody (27) (10 μg of IgG protein) and 70 μl of Protein A-Sepharose CL-4B suspension (50%, v/v). The immune complexes immobilized on Protein A-Sepharose CL-4B were washed five times with lysis buffer (150 mM NaCl, 0.5 mM EGTA, 0.1 mM dithiothreitol, 20 mM Tris·HCl buffer, pH 7.4) and mixed with 20 μl of SDS solution (60 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue) (37). The samples were boiled for 3 min and then centrifuged at 15,000 × *g* for 5 min. The aliquots of the supernatants were analyzed in 10% acrylamide gels by SDS-PAGE.

Statistical analysis. Data are expressed as mean ± standard error. The statistical evaluation of the data was performed with a linear regression analysis and analysis of variance. When a significant *F* value was found by analysis of variance, Scheffé's test for multiple comparisons was carried out to identify differences among groups.

Results

Time-dependent increases in Ca^{2+}-independent activity of CaM kinase II, catecholamine secretion, and tyrosine hydroxylase activity stimulated by acetylcholine in cultured bovine adrenal medullary cells. Fig. 1A shows the time course of the increase in the Ca^{2+}-independent activity, a useful index of activation of CaM kinase II, induced by acetylcholine in cultured bovine adrenal medullary cells. The Ca^{2+}-independent activity increased rapidly at 20 sec after stimulation and reached a plateau at 3–5 min. Fig. 1, B and C, shows the time courses of the increases in catecholamine secretion and tyrosine hydroxylase activity, respectively, produced by the same acetylcholine stimulation. The time dependence of the Ca^{2+}-independent activity was well correlated with those of catecholamine secretion ($r = 0.99$, $p < 0.0001$) and tyrosine hydroxylase activity ($r = 0.99$, $p < 0.0001$).

When the activation of tyrosine hydroxylase was assayed at both subsaturating (0.25 mM) and saturating (1.0 mM) concentrations of pteridine cofactor, the degree of activation of enzyme was almost same (data not shown), suggesting that acetylcholine stimulates the activity of tyrosine hydroxylase by an increase in the V_{max} of the enzyme. This interpretation is in agreement with the data obtained by us (11) and Chalfie *et al.* (38) in rat pheochromocytoma PC-12 cells.

Concentration-dependent increases in Ca^{2+}-independent activity of CaM kinase II, catecholamine secretion, and tyrosine hydroxylase activity stimulated by acetylcholine. Acetylcholine increased the Ca^{2+}-independent activity in a concentration-dependent manner (Fig. 2A). A significant increase in the Ca^{2+}-independent activity was detectable with 10 μM acetylcholine, and the maximum effect (a 2.2-fold increase) was observed with 0.3 mM (Fig. 2A). This concentration-dependent increase was also correlated with those of catecholamine secretion ($r = 0.94$, $p < 0.001$) and tyrosine hydroxylase activity ($r = 0.98$, $p < 0.0001$) (Fig. 2, B and C).

Extracellular Ca^{2+} dependence of acetylcholine-stimulated increases in Ca^{2+}-independent activity of CaM kinase II, catecholamine secretion, and tyrosine hydroxylase activity. We examined the effect of extracellular Ca^{2+} removal on the Ca^{2+}-independent activity of CaM kinase II stimulated by acetylcholine. Acetylcholine produced a 2-fold increase in the Ca^{2+}-independent activity. When Ca^{2+} was removed from the incubation medium and 0.1 mM EGTA was added to the medium, the acetylcholine-induced increase in the Ca^{2+}-independent activity was completely abolished (Fig. 3A). The acetylcholine-induced catecholamine secretion and activation of tyrosine hydroxylase similarly disappeared with extracellular Ca^{2+} depletion (Fig. 3, B and C).

Effects of cholinergic agonists and antagonists on

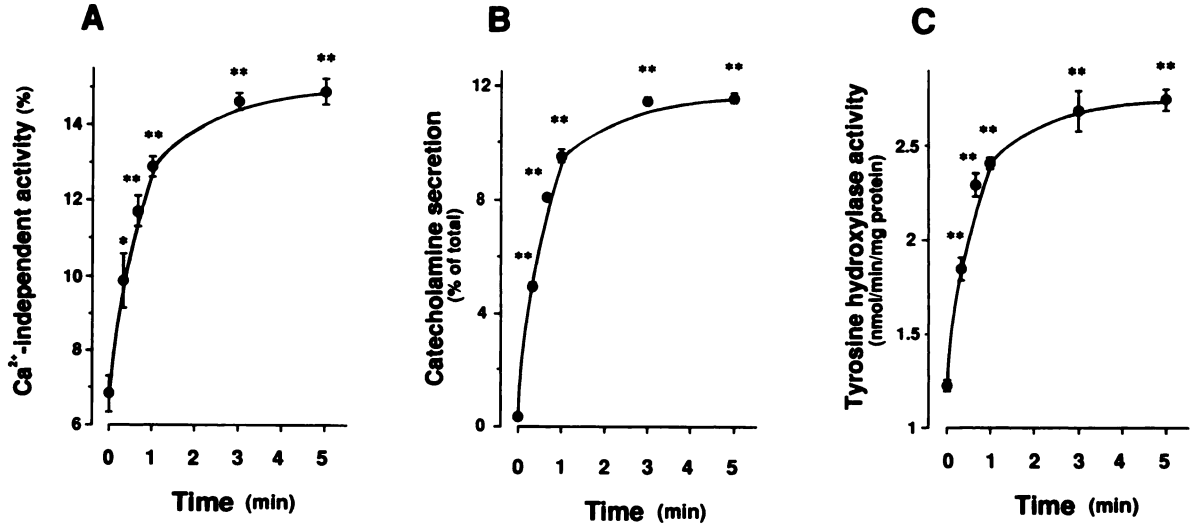

Fig. 1. Time courses of increases in Ca^{2+}-independent activity of CaM kinase II (A), catecholamine secretion (B), and tyrosine hydroxylase activity (C) stimulated by acetylcholine in cultured adrenal medullary cells. The cells were treated with 0.3 mM acetylcholine in 1 ml of Krebs-Ringer phosphate buffer at 37° for the indicated periods, and then the CaM kinase II activity in the cytosol, catecholamines secreted in the reaction medium, and tyrosine hydroxylase activity in the cytosol were assayed as described in Experimental Procedures. Data are expressed as mean ± standard error of three to seven samples. *, $p < 0.05$; **, $p < 0.01$, compared with 0 min.

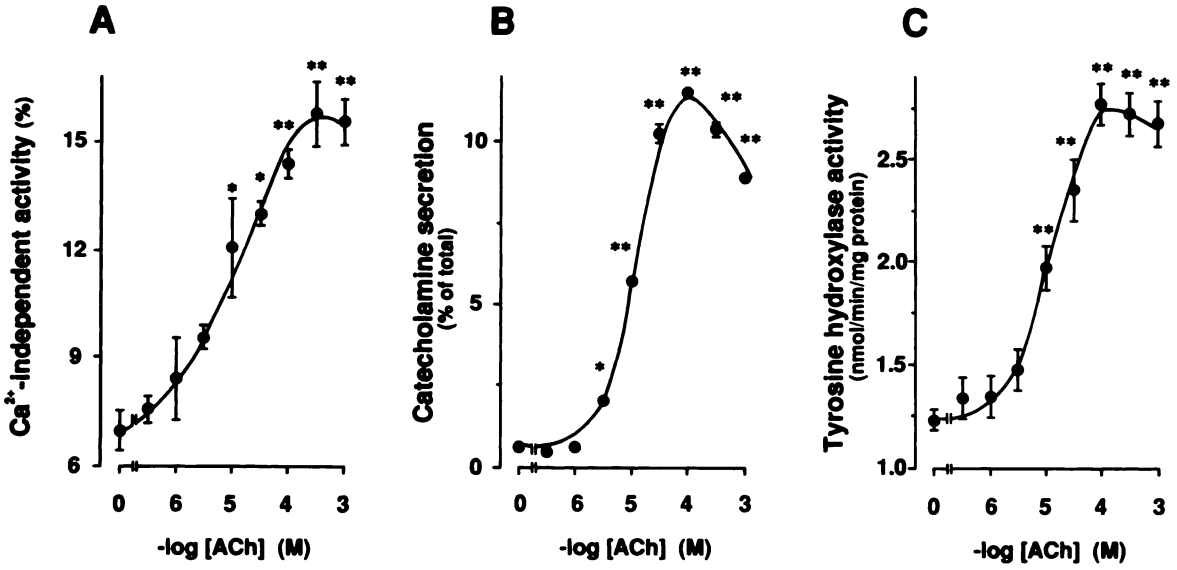

Fig. 2. Concentration-response curves for Ca^{2+}-independent activity of CaM kinase II (A), catecholamine secretion (B), and tyrosine hydroxylase activity (C) stimulated by acetylcholine. The cells were treated with the indicated concentrations of acetylcholine (*ACh*) at 37° for 3 min. Data are expressed as mean ± standard error of three to seven samples. *, $p < 0.05$; **, $p < 0.01$, compared with 0 M.

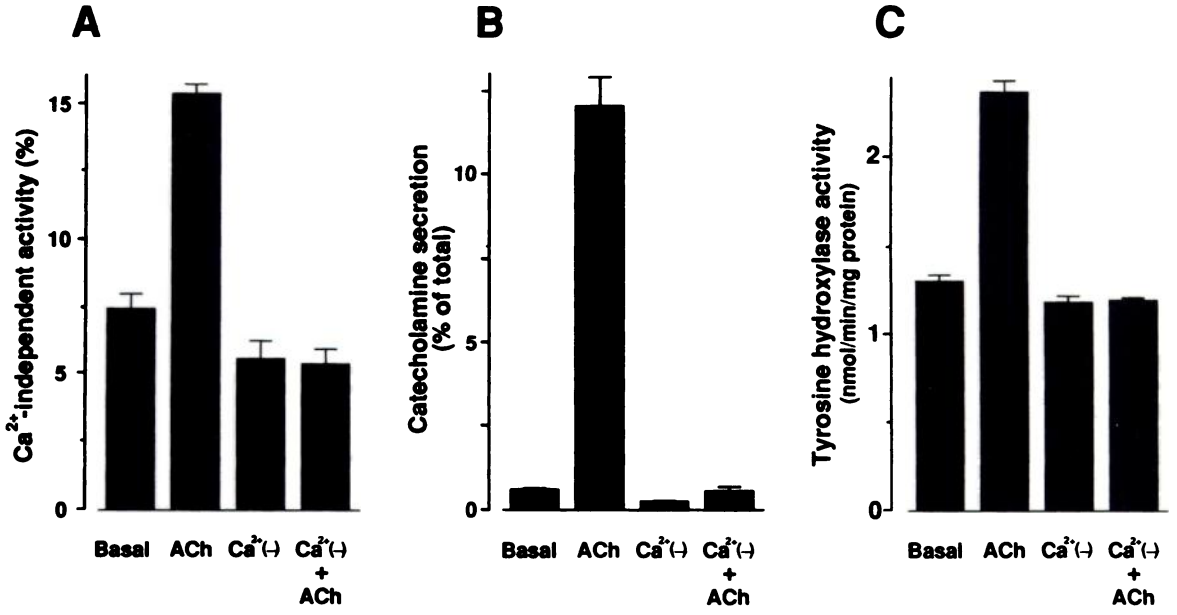

Fig. 3. Effects of extracellular Ca^{2+} removal on Ca^{2+}-independent activity of CaM kinase II (A), catecholamine secretion (B), and tyrosine hydroxylase activity (C) stimulated by acetylcholine. To deplete extracellular Ca^{2+}, Ca^{2+} was removed from the Krebs-Ringer phosphate buffer, pH 7.4, and 0.1 mM EGTA was added to it. Reactions were carried out with or without 0.1 mM acetylcholine at 37° for 3 min in the presence or absence of extracellular Ca^{2+}. Data are expressed as mean ± standard error of three to six samples. *ACh*, acetylcholine; *Ca^{2+}(−)*, Ca^{2+}-free Krebs-Ringer phosphate buffer.

Ca^{2+}-independent activity of CaM kinase II. Acetylcholine receptors are known to include the muscarinic and nicotinic receptors in bovine adrenal medullary cells (28). To study which receptor type contributes to the acetylcholine-increased Ca^{2+}-independent activity of CaM kinase II, we used cholinergic agonists (Fig. 4A) and antagonists (Fig. 4B). Muscarine produced no significant increase (Fig. 4A). In contrast, nicotine markedly increased the activity, and the effect of nicotine was comparable to that of acetylcholine (Fig. 4A). Atropine, an antagonist of the muscarinic acetylcholine receptor, produced no effect on the acetylcholine-induced increase in the activity (Fig. 4B). Hexamethonium, an antagonist of the nicotinic acetylcholine receptor, inhibited the acetylcholine-induced increase to basal levels (Fig. 4B).

Effects of various secretagogues on Ca^{2+}-independent activity of CaM kinase II. Various secretagogues were examined to study the mechanisms of activation of CaM kinase II in the cells. Bradykinin, which mobilizes intracellular Ca^{2+} (39), had no effect on the Ca^{2+}-independent activity (Table 1). Histamine, which mobilizes both intracellular and extracellular Ca^{2+} (40), ionomycin, calcium ionophore, and 56 mM K^+, an activator of voltage-dependent Ca^{2+} channels, significantly increased the activity (Table 1). Veratridine, which activates the voltage-dependent Na^+ channels and causes membrane depo-

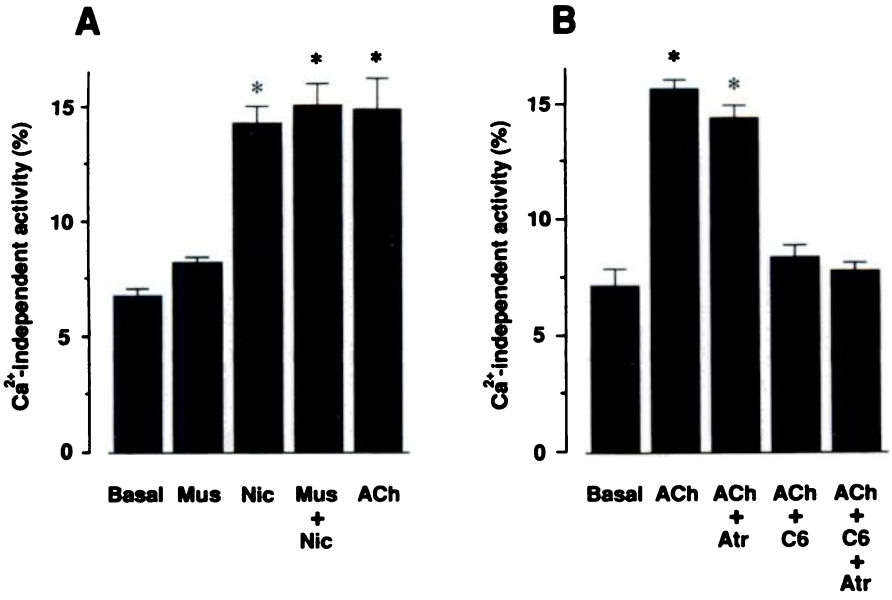

Fig. 4. Effects of cholinergic agonists (A) and antagonists (B) on Ca^{2+}-independent activity of CaM kinase II. The cells were incubated with the indicated agonists or antagonists, including muscarine, nicotine, acetylcholine, atropine, and hexamethonium, at 37° for 3 min. The CaM kinase II activity in the cytosol was then assayed as described in Experimental Procedures. Data are expressed as mean ± standard error of four to six samples. *, $p < 0.01$, compared with basal. *Mus*, 0.1 mM muscarine; *Nic*, 0.1 mM nicotine; *ACh*, 0.1 mM acetylcholine; *Atr*, 1 μM atropine; *C6*, 1 mM hexamethonium.

TABLE 1

Effects of various secretagogues on Ca^{2+}-independent activity of CaM kinase II

The cells were incubated with bradykinin, histamine, or ionomycin for 10 min or with K^+, veratridine, or tetrodotoxin for 5 min (four experiments). The CaM kinase II activity in the cytosol fraction was then assayed. Data are expressed as mean ± standard error.

	Ca^{2+}-independent activity of CaM kinase II
	%
Basal	8.20 ± 0.35
Bradykinin, 0.1 mM	9.88 ± 0.64
Histamine, 0.1 mM	10.98 ± 0.60[a]
Ionomycin, 10 μM	14.09 ± 0.76[b]
Basal	7.51 ± 0.25
K^+, 56 mM	15.18 ± 0.25[b]
Veratridine, 0.1 mM	11.03 ± 0.33[b]
Veratridine, 0.1 mM + tetrodotoxin, 1 μM	7.60 ± 0.31

[a] $p < 0.05$, compared with basal level.
[b] $p < 0.01$, compared with basal level.

larization (41), also significantly increased the Ca^{2+}-independent activity, and tetrodotoxin, a blocker of the voltage-dependent Na^+ channels (41), inhibited the veratridine-induced increase (Table 1).

Phosphorylation of a 50-kDa protein stimulated by acetylcholine in the cells. Direct evidence of CaM kinase II activation in intact cells is provided by demonstration of the autophosphorylation of the enzyme. ^{32}P-Labeled adrenal medullary cells were stimulated with 0.3 mM acetylcholine and the supernatants were immunoprecipitated with affinity-purified anti-CaM kinase II antibody. The immunoprecipitates were then analyzed by SDS-PAGE, followed by autoradiography (Fig. 5). Acetylcholine (0.3 mM) stimulated the phosphorylation of a 50-kDa protein in the cells, with a 66% increase over control (Fig. 5, *lane 2*).

In our recent study (19), the autophosphorylated subunit of the CaM kinase II isoenzyme isolated from adrenal medullary cells migrated to the same position in SDS-PAGE as did the 50-kDa protein. In view of the specificity of the anti-brain CaM

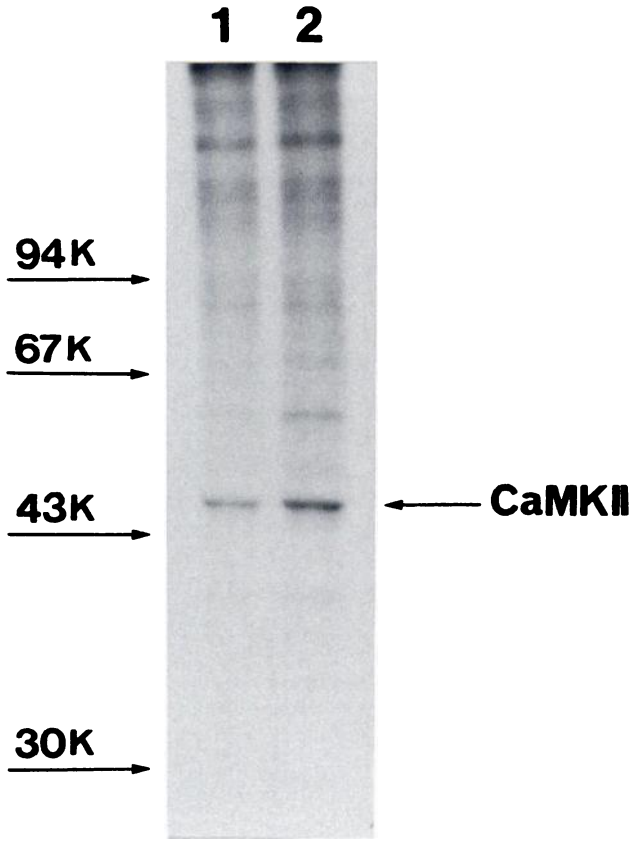

Fig. 5. Phosphorylation of a 50-kDa protein stimulated by acetylcholine in cultured bovine adrenal medullary cells. The cells were labeled at 37° for 1 hr in 1 ml of Krebs-Ringer-HEPES buffer, pH 7.4, containing [^{32}P]P_i (0.5 mCi/ml). The ^{32}P-labeled cells were stimulated with 0.3 mM acetylcholine at 37° for 3 min, and the supernatants were immunoprecipitated with affinity-purified anti-CaM kinase II antibody. The immunoprecipitates were then analyzed by SDS-PAGE, followed by autoradiography. *Lane 1*, control; *lane 2*, acetylcholine. *CaMKII*, CaM kinase II.

kinase II antibody (22, 27), the 50-kDa protein (Fig. 5) was concluded to be the subunit of the CaM kinase II isoenzyme in bovine adrenal medullary cells. The results suggest that CaM kinase II in intact adrenal medullary cells is autophosphorylated, with a concomitant increase in the Ca^{2+}-independent activity, in response to the stimulation with acetylcholine.

Discussion

Recently, we have isolated two Ca^{2+}/calmodulin-dependent protein kinases from cultured bovine adrenal medullary cells (19). One of the kinases has the following characteristic properties: (i) a high molecular mass (650 kDa) for the holoenzyme on a gel filtration column, (ii) a subunit structure of about 50 kDa, (iii) autophosphorylation, (iv) broad substrate specificity, and (v) immunoprecipitation with the anti-brain CaM kinase II antibody. From these results, we have concluded that the isoenzyme of CaM kinase II exists in this cellular system (19). Based on these findings, we have further examined the functional linkage of CaM kinase II in the cells.

In the present study, we have demonstrated that acetylcholine causes a time- and concentration-dependent increase in the Ca^{2+}-independent activity of CaM kinase II (Figs. 1 and 2). The increase in the Ca^{2+}-independent activity has been regarded as an index of CaM kinase II activation in various intact tissues, such as hippocampal slices (21, 25), brain synaptosomes (13), cerebellar granule cells (20), GH_3 pituitary cells (24), pheochromocytoma PC-12 cells (23), and neuroblastoma × glioma hybrid NG108–15 cells (26).

Stimulatory effect of acetylcholine on CaM kinase II activity through nicotinic acetylcholine receptor-mediated influx of Ca^{2+}. In bovine adrenal medulla, nicotinic acetylcholine receptors are involved in the secretion of catecholamines and muscarinic acetylcholine receptors are not (28). In the present study, nicotine but not muscarine is able to substitute for acetylcholine in activation of CaM kinase II, and the acetylcholine-induced activation is completely blocked by hexamethonium but not by atropine (Fig. 4). The present results indicate that mainly the nicotinic acetylcholine receptor contributes to the activation of CaM kinase II.

Acetylcholine is known to evoke acetylcholine receptor-gated Na^+ influx and to cause membrane depolarization, leading to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (41). In the present study, acetylcholine produces a stimulatory effect on CaM kinase II activity (Figs. 1 and 2), which is abolished by extracellular Ca^{2+} depletion (Fig. 3). CaM kinase II activation is also observed with agents that cause an influx of Ca^{2+}, such as histamine (40), ionomycin (41), 56 mM K^+ (41), and veratridine (41) (Table 1). On the other hand, bradykinin (39) and muscarine (28), both of which mobilize Ca^{2+} from intracellular Ca^{2+} stores, produce no significant increase in activity (Fig. 4A; Table 1). In our recent study (19), there was a temporal correlation between 56 mM K^+-evoked increases in $^{45}Ca^{2+}$ influx and in CaM kinase II activity ($r = 0.97$, $p < 0.001$), and the two were concomitantly attenuated by the addition of 20 mM $MgSO_4$, an inhibitor of voltage-dependent Ca^{2+} channels. These results suggest that Ca^{2+} influx is more important than Ca^{2+} release from intracellular stores for activation of CaM kinase II, at least in our preparation. This interpretation is well supported by the findings that acetylcholine and high K^+ evoke large increases of cytosolic free Ca^{2+} concentrations, whereas bradykinin and muscarine produce only small increases, in bovine adrenal medullary cells (39, 42). The present results also suggest that acetylcholine stimulates CaM kinase II activity through the nicotinic acetylcholine receptor-mediated influx of Ca^{2+}.

Close relationship between CaM kinase II activation and catecholamine secretion or tyrosine hydroxylase activation stimulated by acetylcholine. To elucidate the functional role of CaM kinase II in the adrenal medulla, the relationship between CaM kinase II activation and catecholamine secretion or tyrosine hydroxylase activation stimulated by acetylcholine has been examined. The time course and concentration-response curves for Ca^{2+}-independent activity are closely correlated (almost identical) with those for catecholamine secretion and tyrosine hydroxylase activity (Figs. 1 and 2). The acetylcholine-induced activation of CaM kinase II, as well as catecholamine secretion and tyrosine hydroxylase activation, is completely abolished by removal of extracellular Ca^{2+} (Fig. 3). To our knowledge, this is the first report that shows a good correlation of the activation of CaM kinase II with the cellular functions of intact adrenal medullary cells.

The present study does not demonstrate whether CaM kinase II plays a crucial role in the regulation of catecholamine secretion and synthesis. However, four lines of previously reported evidence suggest the functional involvement of CaM kinase II in adrenal medulla. First, an isozyme of CaM kinase II exists in cultured bovine adrenal medullary cells (19). Second, CaM kinase II phosphorylates tyrosine hydroxylase and activates it in the presence of activator protein *in vitro* (43, 44). Third, the tryptic peptide map of tyrosine hydroxylase phosphorylated in response to carbachol or high-K^+ depolarization in PC-12 cells (15, 16) and cultured adrenal medullary cells (17, 18) is similar to that of the enzyme phosphorylated by CaM kinase II *in vitro*. Finally, CaM kinase II phosphorylates several proteins of chromaffin granule membranes, as well as a novel endogenous substrate (70 kDa) from bovine adrenal medullary cells (19). In our laboratory, a knockout experiment using an antisense against CaM kinase II is now ongoing and will provide more important information for the study.

In conclusion, the physiological stimulant acetylcholine activates CaM kinase II via activation of nicotinic acetylcholine receptors in cultured adrenal medullary cells. Alterations of CaM kinase II activity are in parallel with those of catecholamine secretion and tyrosine hydroxylase activity in the cells.

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