Biochem. J. (1989) 264, 589–596 (Printed in Great Britain)

Phosphorylation of myosin light chain from adrenomedullary chromaffin cells in culture

Luis M. GUTIERREZ,* Maria J. HIDALGO,* Mercedes PALMERO,* Juan J. BALLESTA,* Juan A. REIG,* Antonio G. GARCIA† and Salvador VINIEGRA*‡

*Departamento de Neuroquímica, Facultad de Medicina, Universidad de Alicante, Alicante, and †Departamento de Farmacologia, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain

The myosin-light-chain (MLC) phosphorylation accompanying catecholamine release in chromaffin cells was investigated with the objective of assessing the possible role of this contractile protein in catecholamine secretion. The electrophoretic characteristics of adrenomedullary MLC were determined by immunochemical techniques using two different specific antibodies. The identified 22 kDa phosphoprotein was mainly present in the cytosol, as demonstrated by ultracentrifugation and immunocytochemical analysis. A part of this protein was located on, or close to, the plasma membrane. Cell stimulation by secretagogues resulted in a Ca^{2+}-dependent ^{32}P incorporation into MLC, the time course of this process being related to catecholamine release. These findings were supported by a two-dimensional gel-electrophoretic analysis by which means this protein was resolved into two acidic forms. A role for Ca^{2+}-calmodulin and Ca^{2+}-phospholipid kinases in adrenomedullary MLC phosphorylation is reported. The results obtained suggest a regulatory role for such a protein in the underlying exocytotic event.

INTRODUCTION

Catecholamine release from adrenomedullary chromaffin cells is triggered by a rise in the cytosolic Ca^{2+} concentration (Knight & Kesteven, 1983) as a consequence of an increased permeability of the plasma membrane to external Ca^{2+} caused by acetylcholine-receptor stimulation (Douglas & Rubin, 1961). The sequence of events that occur in chromaffin cells between Ca^{2+} entry and catecholamine secretion remains poorly understood. A possible role for protein phosphorylation in stimulus–secretion coupling in several systems, including nervous tissue (Krueger *et al.*, 1977; De Lorenzo & Freedman, 1978), mast cells (Hempstead *et al.*, 1981), platelets (Haslam & Lynham, 1977; Kaibuchi *et al.*, 1983), chromaffin cells (Amy & Kirshner, 1981) and other cell types has been suggested.

In adrenomedullary tissue, studies of protein phosphorylation have included the effect of secretagogues on ^{32}P incorporation into proteins in monolayer cultures. These phosphoproteins have been quantified by densitometry of autoradiograms obtained by one- (Côté *et al.*, 1986) or two-dimensional (Gutierrez *et al.*, 1988) gel electrophoresis. Other studies have been carried out on digitonin-permeabilized cells (Lee & Holz, 1986) as well as phosphorylation *in vitro* of different subcellular fractions (Burgoyne & Geisow, 1982; Wise & Costa, 1985).

Some of the phosphoproteins reported in these previous studies might be implicated in the regulation and/or triggering of the exocytotic mechanism. In this context we have identified, with immunochemical techniques, one of the myosin light chains (MLCs) in bovine adrenal chromaffin cells. We demonstrate here that this protein was Ca^{2+}-dependently phosphorylated during catecholamine release induced by different secretagogues and that the time courses of these two processes are related. We also studied the possible implication of Ca^{2+}-diacylglycerol-activated (protein kinase C) and Ca^{2+}-calmodulin kinases in the phosphorylation of MLC in cultured adrenomedullary cells. Our experiments suggest that phosphorylation of adrenomedullary MLC that occurs during secretion might play a modulatory role in this physiological event similar to that found in processes like actin–myosin interactions in different cell types (Adelstein & Eisenberg, 1980) or in platelet activation (Nishikawa *et al.*, 1980; Naka *et al.*, 1983).

MATERIALS AND METHODS

Materials

Carrier-free [^{32}P]orthophosphate (25–40 mCi/ml) was purchased from Amersham International. Anti-myosin IgG was from ICN Immunochemicals and peroxidase-labelled affinity-purified antibody to rabbit IgG from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). All reagents were of analytical grade from Sigma or Merck.

Cell culture

Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion (Almazan *et al.*, 1984), isolated from debris and erythrocytes with a Percoll gradient and plated, at a density of 250000 cells/cm^2, on uncoated plastic culture dishes in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) foetal-calf serum, 10 μM-cytosine arabinoside, penicillin

Abbreviations used: MLC, myosin light chain; K/H, Krebs/Hepes (solution); TFP, trifluoperazine; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline (composition is given in the text); BSA, bovine serum albumin; MLCK, myosin-light-chain kinase.

‡ To whom correspondence and reprint requests should be sent.

(25000 i.u./litre) and streptomycin (25 mg/litre). Cells were used between days 3 and 6 after plating.

Phosphorylation of cell proteins in response to secretory stimuli

Culture medium was removed and cells washed three times with Krebs/Hepes (K/H) solution [134 mM-NaCl/4.7 mM-KCl/1.2 mM-KH_2PO_4/1.2 mM-$MgCl_2$/2.5 mM-$CaCl_2$/15 mM-Hepes/11 mM-glucose/L-ascorbate (100 mg/litre), equilibrated with pure O_2 and the pH adjusted to 7.4]. After the washing period, monolayers were incubated for 1 h at 37 °C in K/H solution lacking KH_2PO_4, containing [^{32}P]orthophosphate (1 mCi/ml), then quickly washed three times with fresh K/H solution and incubated for different periods between 15 and 300 s with one of the following solutions: K/H (basal conditions); K/H 59 mM-K^+ (70.9 mM-NaCl, 57.8 mM-KCl) with or without addition of 2.5 mM-$CaCl_2$, K/H with 100 μM-acetylcholine chloride and K/H 59 mM-K^+ containing the Ca^{2+}-channel activator Bay-K-8644 at 1 μM. This drug was previously preincubated for 15 min at the same concentration in K/H basal medium. After the stimulation period, media were collected in order to determine the amount of catecholamine secreted. Immediately thereafter, cells were disrupted with SDS buffer (0.1% SDS/5% β-mercaptoethanol/1% Nonidet P40 in 50 mM-Tris/HCl buffer, pH 7.5) or scraped into buffer for the immunoprecipitation procedure (0.15 M-NaCl/50 mM-Tris/HCl, pH 7.5).

Effect of trifluoperazine on high-K^+-evoked secretion and MLC phosphorylation

Trifluoperazine (TFP) at 0.5 μM in K/H 59 mM-K^+ was employed in order to check its effect on catecholamine release and MLC phosphorylation induced by high K^+ (59 mM) during 1 and 5 min periods. The drug was also preincubated in K/H for 15 min before stimulation. Then 0.5 ml of K/H 59 mM-K^+ medium with or without TFP was added to monolayers. At the end of stimulation period, media were collected to determine the amount of catecholamine released, and cells were lysed with SDS buffer to analyse the phosphoproteins by electrophoresis.

Effect of phorbol 12-myristate 13-acetate (PMA) on secretion and MLC phosphorylation induced by the Ca^{2+} ionophore A23187

Monolayers that had been previously incubated with [^{32}P]orthophosphate were exposed to the Ca^{2+} ionophore A23187 at 1 μM in K/H solution for 1, 5, 15 and 30 min periods in the presence or absence of 0.1 μM-PMA. Media were collected, and the cells were lysed as described above.

Electrophoresis

Electrophoresis was performed, as described by Laemmli (1970), in SDS/12.5%-(w/v)-polyacrylamide/0.1% bisacrylamide slab gels. Dry gels were autoradiographed for 3–5 days at −70 °C using Kodak X-Omat AR films. The intensity of bands in X-ray films were measured by scanning in a densitometer. The ratio of the area of each peak above the baseline to the total area was determined to correct variations in the quantity of sample applied on the gels. Two-dimensional gel electrophoresis was carried out as described by Bravo (1984), the first dimension by using a range of Ampholines between pH 3.5 and 10.0, and the second dimension by using SDS/polyacrylamide-gel electrophoresis as described above. The pH gradient obtained in isoelectric focusing was determined as described by Lee & Holz (1986), giving a linear gradient between pH 4.5 and 6.5.

Pre-stained standards with molecular masses in the range 14.3–200 kDa were from Amersham International or Bethesda Research Laboratories.

Immunochemical techniques

Immunoblotting. Transfer was performed as described by Towbin *et al.* (1979), and prestained molecular-mass standards were used to check transfer efficiency. Nitrocellulose sheets were shaken in TBS [Tris-buffered saline (20 mM-Tris/HCl/0.5 M-NaCl, pH 7.5)] containing 3% bovine serum albumin (BSA) for 1 h before overnight incubation with anti-myosin IgG or rabbit anti-(human heart MLC) serum (1:250 dilution in the same buffer). Then sheets were washed four times in TBS before incubation with IgG-conjugated horseradish peroxidase (1 μg/ml in TBS with 3% BSA) during 2 h. After gentle washing in TBS, immunostaining, with 4-chloro-1-naphthol as a dye, was performed.

Immunoprecipitation. Scraped cells in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.5, were sonicated and centrifuged at 100000 *g* for 30 min. The pellet and supernatant fractions obtained by this procedure were used to define the location of ^{32}P-incorporated polypeptides as cytosolic or non-cytosolic proteins. Supernatant aliquots (75 μl) were incubated overnight at

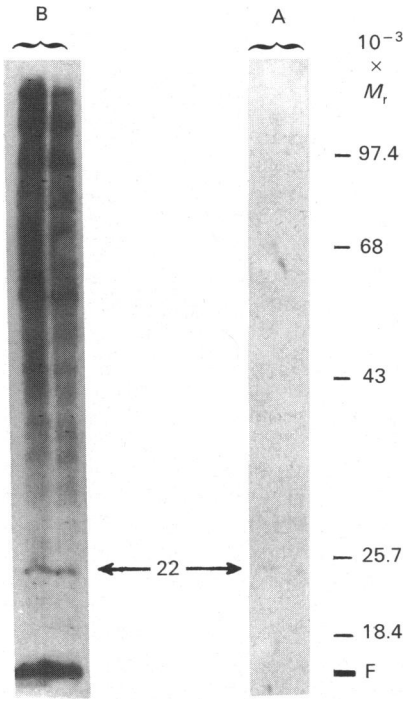

Fig. 1. Immunoblotting using rabbit anti-(bovine myosin) serum (A) of total proteins from chromaffin cells incubated with [^{32}P]orthophosphate

B is an autoradiogram of phosphoproteins transferred to a nitrocellulose sheet. The arrows (← →) indicate the specific immunostain and the associated 22 kDa phosphoprotein band.

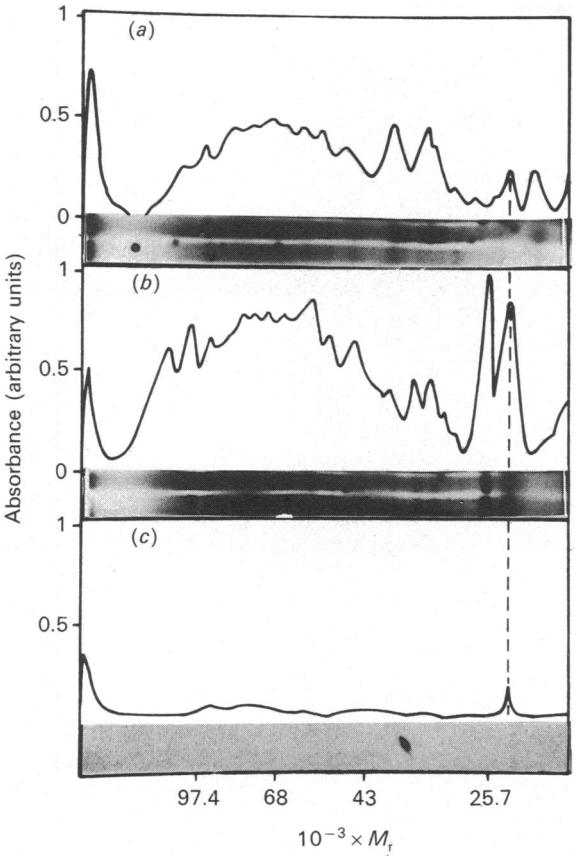

Fig. 2. Autoradiogram and densitometric scan of phosphate-accepting proteins from the 100000 *g* pellet (*a*) and the supernatant (*b*) from sonicated adrenomedullary chromaffin cells

(*c*) shows a 22 kDa phosphoprotein immunoprecipitated by anti-(human MLC) antibody in the cytosolic fraction. The broken line indicates the position of the MLC band (22 kDa).

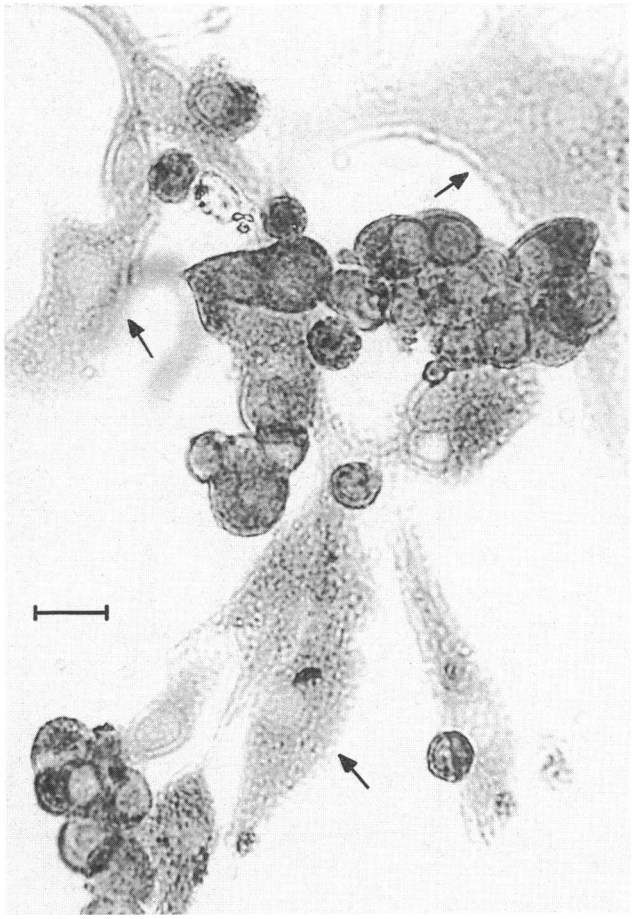

Fig. 3. Immunocytochemical staining of cultured chromaffin cells with anti-(human MLC) serum employing the anti-(rabbit IgG)–peroxidase method

Aggregated chromaffin cells were stained more strongly than fibroblasts (indicated by arrows) and their plasma membranes were apparently more defined by staining. The bar represents 20 μm.

4 °C with the antibodies mentioned above (1:30 dilution). After that, 50 μl of 10% (w/v) of protein A–Sepharose diluted in the same buffer was added, the mixture shaken for 1 h, and precipitates (obtained by centrifugation at 10000 *g*) were briefly washed four times with the same buffer. Finally, before electrophoresis the immunoprecipitates were heated for 5 min at 100 °C in SDS buffer.

Immunocytochemical technique. Cultured cells were washed with 0.1% BSA in TBS buffer before being fixed and permeabilized with pure methanol for 15 min at −20 °C. After that, cells were washed again with the same buffer and incubated overnight at 4 °C with the antibodies mentioned above at 1:50 dilution. Controls were performed with rabbit serum. Monolayer cultures were gently washed and incubated for 1 h at room temperature with anti-rabbit IgG-conjugated horseradish peroxidase at 1:100 dilution in TBS buffer with 1% BSA. Finally, cells were washed with the same buffer and immunostaining was performed with diaminobenzidine (50 μg/100 ml) in 50 mM-Tris/HCl buffer, pH 7.5, with H_2O_2 at a final concentration of 0.3%.

Other methods

Catecholamines were determined by the fluorimetric assay of Shellenberger & Gordon (1971) and protein quantification was performed by the method of Bradford (1976).

RESULTS

Immunological characterization of bovine adrenal medulla MLC

The electrophoretic characterization of bovine adrenomedullary MLC was achieved by immunoblotting with two different antibodies against this protein.

Total cell protein from chromaffin cell monolayer cultures was subjected to electrophoresis after incubation with [^{32}P]orthophosphate. Resolved proteins were transferred to a nitrocellulose sheet and incubated with rabbit IgG against bovine myosin. The specific immunostain corresponded to a band with an associated molecular mass of 22±1 kDa (Fig. 1, track A) that was closely related to a phosphoprotein band with the same relative mobility present in the autoradiograph of the nitrocellulose sheet (Fig. 1, track B). Identical results were obtained by using rabbit anti-(human heart MLC) antiserum.

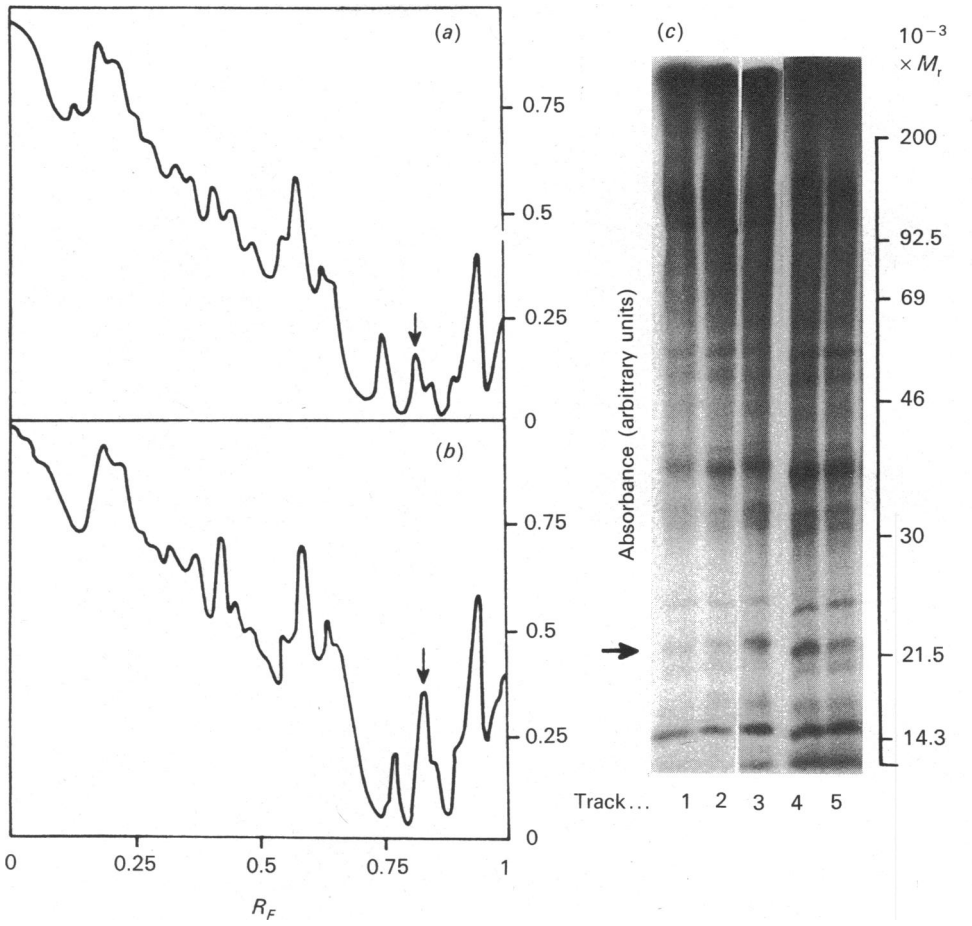

Fig. 4. Autoradiograms (*c*) of phosphoproteins resolved by electrophoresis of chromaffin cells incubated under different stimulation conditions for 1 min

Stimulation conditions were: K/H (track 1), K/H 59 mM-K^+ without Ca^{2+} (track 2), K/H 59 mM-K^+ (track 3), K/H 59 mM-K^+ with 1 μM-Bay-K-8644 (track 4) and K/H with 100 μM-acetylcholine chloride (track 5). The densitometric tracing of the autoradiogram relating to K/H is shown in (*a*) and that relating to K/H 59 mM-K^+ with 1 μM-BAY-K-8644 in (*b*). The arrow indicates the position of the MLC band. The area under the MLC peak in relation to the total area in the densitogram was used as a measure of MLC phosphorylation.

To determine the subcellular distribution of this phosphoprotein, after the incorporation of ^{32}P into monolayer cultures the cells were sonicated and centrifuged at 100000 *g* for 30 min. Two different subcellular fractions were obtained: a pellet consisting of organelles and membrane systems, and a cytosolic supernatant. The autoradiographic pattern of each fraction resolved by electrophoresis and the corresponding densitometric scans are shown in Fig. 2. The 22 kDa phosphoprotein is predominantly located in the cytosolic component of chromaffin cells (Fig. 2*b*). A fraction of this protein was detected in the 100000 *g* pellet (Fig. 2*a*). Additional proof of the identity of this band as chromaffin-cell MLC and of its presence in the cytosol was sought. By using the antibodies mentioned above, a ^{32}P-incorporating protein was immunoprecipitated from the cytosolic fraction (Fig. 2*c*). The relative mobility and associated molecular mass of this protein were coincident with those obtained in the immunoblotting experiments.

The cellular distribution of antigenic sites for MLC and myosin antibodies were studied by an immunocytochemical technique employing IgG-labelled peroxidase. The MLC location obtained by this technique is shown in Fig. 3. A great density of stain was present in chromaffin cells, in contrast with that observed in fibroblasts also present in the culture. The chromaffin-cell stain was mainly distributed in the cytosol, and a part of this was localized in, or close to, the plasma membranes. The lower density of stain observed in the nucleus probably corresponds to the stain of the cytoplasmic part located over it. Both types of antibodies showed a similar cell-distribution pattern. Controls made with normal rabbit serum were characterized by the absence of stain.

Induction of MLC phosphorylation by different stimuli

We decided to find out whether secretory stimuli were able to modify the extent of MLC phosphorylation in adrenomedullary chromaffin cells in culture. Assays were carried out by using high K^+ or acetylcholine as secretory agents. Monolayers of chromaffin cells were exposed for periods between 15 and 300 s to the stimulus; thereafter the amount of catecholamines secreted and the amount of ^{32}P incorporated into the MLC band were determined.

A representative autoradiogram of phosphoproteins resolved by SDS/polyacrylamide gels is shown in Fig.

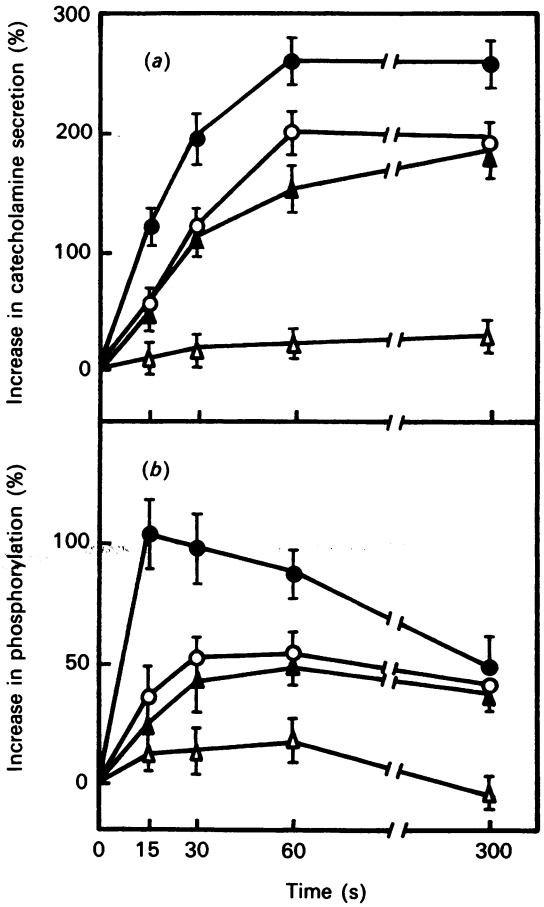

Fig. 5. Time-course of catecholamine secretion (*a*) and MLC phosphorylation (*b*) under different stimulation conditions

Stimulation conditions were: K/H 59 mM-K^+ (○), K/H 59 mM-K^+ without Ca^{2+} added (△), K/H 59 mM-K^+ with 1 μM-BAY-K-8644 (●) and K/H basal with 100 μM-acetylcholine chloride (▲). Results are expressed as percentage increases over control (5.9 mM-K^+) value, which was 100 ng of catecholamine/5×10^5 cells, and are given as means ± S.E.M. for four experiments.

4(*c*). The density of the MLC band was increased when cells were exposed for 1 min to different secretory stimuli such as K/H 59 mM-K^+ (track 3); K/H 59 mM-K^+ with 1 μM-BAY-K-8644 (track 4) or 100 μM-acetylcholine (track 5), in relation to the phosphorylation level shown in basal (track 1) or K/H 59 mM-K^+ in the absence of added Ca^{2+} (track 2). In the same Figure the densitometric tracings of autoradiograms under basal K/H (*a*) and K/H 59 mM-K^+ with BAY-K-8644 (*b*) conditions shows the maximum differences in MLC phosphorylation observed in the experiment and measured by peak area integration.

As Fig. 5(*a*) shows, when cells were incubated in a depolarizing stimulus like K/H 59 mM-K^+, catecholamine secretion was increased in a time-dependent manner for the first 1 min, reaching a plateau of a 200% increment over the control in K/H 5.9 mM-K^+ (100 ng of catecholamine/5×10^5 cells); no further increase was subsequently observed. Under these conditions, the incorporation of ^{32}P into the MLC band (Fig. 5*b*) was increased by 50% during the first 1 min, and a slight dephosphorylation was observed at longer incubation times. The secretory and MLC phosphorylation profiles obtained when cells were incubated with 100 μM-acetylcholine were similar to those obtained with high K^+. The enhancement of these two processes can be prevented by omitting Ca^{2+} in the high-K^+ media.

When a Ca^{2+}-channel activator (Bay-K-8644, at 1 μM) was added to K/H 59 mM-K^+, the secretion was enhanced 1.2-fold over that for the depolarizing stimulus alone. The MLC showed a high level of phosphorylation in the presence of this dihydropyridine at the shorter incubation time. Then a smooth dephosphorylation was observed at longer stimulation periods.

Additionally, two-dimensional gel electrophoresis resolved this 22 kDa phosphoprotein into two spots of close molecular masses and with isoelectric points of 4.9 and 5.1 respectively. The intensity of these two spots after 1 min incubation of monolayers under different stimulus conditions is shown in Fig. 6. In the presence of K/H 59 mM-K^+ (Fig. 6*b*), the intensity of the more acidic polypeptide was enhanced compared with that in basal K/H (Fig. 6*a*) or when no Ca^{2+} was added (Fig. 6*c*). As in one-dimensional analysis, the presence of Bay-K-8644 (Fig. 6*d*) strongly increased the intensity of both spots, especially the acidic one, over the intensity observed under basal-medium conditions (Fig. 6*a*).

Effect of TFP on MLC phosphorylation induced by depolarization

TFP, a calmodulin antagonist, was employed to check the possible mediation of a Ca^{2+} + calmodulin-dependent kinase in the phosphorylation of MLC. At 0.5 μM, TFP showed an inhibitory effect of 30–35% in the K^+-evoked release assayed at 1 and 5 min in cells pre-incubated with [^{32}P]orthophosphate. Under these conditions, a decrease in the amount of ^{32}P incorporated into MLC was detected in relation to that obtained in the absence of TFP. At 1 min incubation, MLC phosphorylation was inhibited by 40% over the K^+-evoked level (Fig. 7), whereas at 5 min the K^+-evoked MLC phosphorylation in the presence of TFP reached the basal level.

Activation of kinase C and MLC phosphorylation

The Ca^{2+} ionophore A23187 at a concentration of 1 μM in K/H buffer was employed for different time periods between 1 and 30 min. As Fig. 8(*a*) shows a low release of catecholamine was observed in the presence of Ca^{2+} ionophore; this secretion was enhanced by addition of 0.1 μM-PMA, reaching a 2-fold increase at 30 min. In the same experiment, the phosphorylation level of MLC in the presence of A23187 was time-independent (Fig. 8*b*). In the presence of the phorbol ester PMA, an increase of 40% over control with A23187 was detected at 30 min.

DISCUSSION

The immunological characterization performed by immunoblot and immunoprecipitation experiments carried out with a specific antibody against bovine myosin and an anti-(human MLC) serum recognized an identical ^{32}P-incorporating protein with a molecular mass of 22 ± 1 kDa, in close concordance with the characteristics described in isolated myosin from bovine adrenal medulla (Trifaró & Ulpian, 1976; Hesketh *et al.*, 1978) for one of

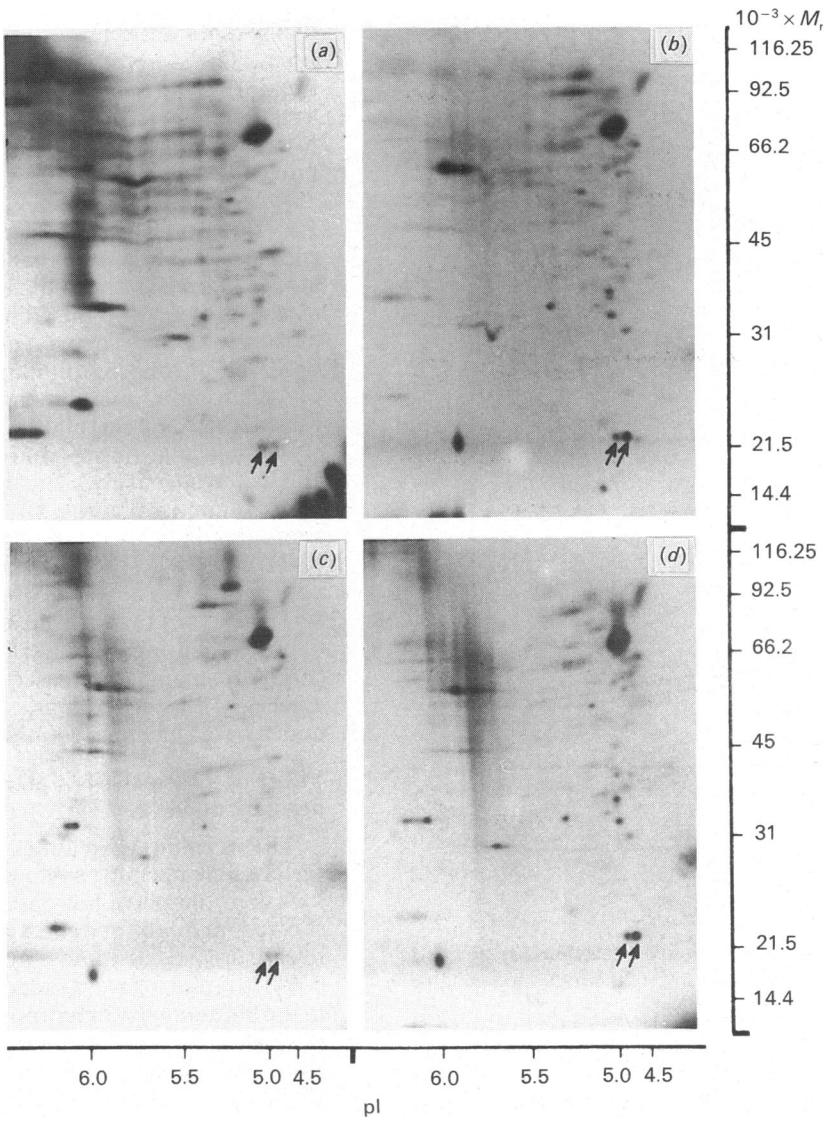

Fig. 6. Two-dimensional (gel electrophoresis/isoelectric focusing) analysis of phosphoproteins

Chromaffin cells were incubated for 1 min with K/H (*a*), K/H 59 mM-K^+ (*b*), K/H 59 mM-K^+ without Ca^{2+} added (*c*) or K/H 59 mM-K^+ in the presence of 1 μM-BAY-K-8644 (*d*). The arrows indicate the resolved MLC forms.

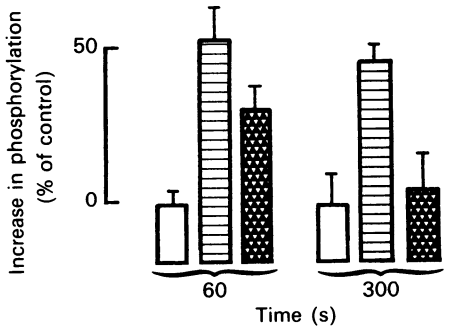

Fig. 7. Effect of TFP on MLC phosphorylation

Monolayer cultures were incubated for 1 or 5 min with K/H (□), K/H 59 mM-K^+ (▤) and K/H 59 mM-K^+ with 0.5 μM-trifluoperazine (▩). Results shown are means ± S.E.M. for four experiments.

the MLCs (22–23 kDa). Similar results were obtained for non-muscle myosin (Clarke & Spudich, 1977).

Although this protein was mainly present in the cytosolic fraction, part of the total content of chromaffin-cell MLC was detected in the pellet constituted by membrane systems.

The subcellular distribution of antigenic sites to these antibodies, performed by the anti-(rabbit IgG)–peroxidase technique, confirmed, by its granular appearance, its cytosolic localization. This was in accordance with data obtained previously from indirect immunofluorescence staining (Trifaró *et al.*, 1978; Aunis *et al.*, 1980). The presence of immunoreactive sites localized in, or close to, the plasma membrane is noteworthy.

Two-dimensional (gel-electrophoresis–isoelectric focusing) analysis of this 22 kDa phosphoprotein resolved two spots with similar molecular masses but different isoelectric points (4.9 and 5.1). These acidic

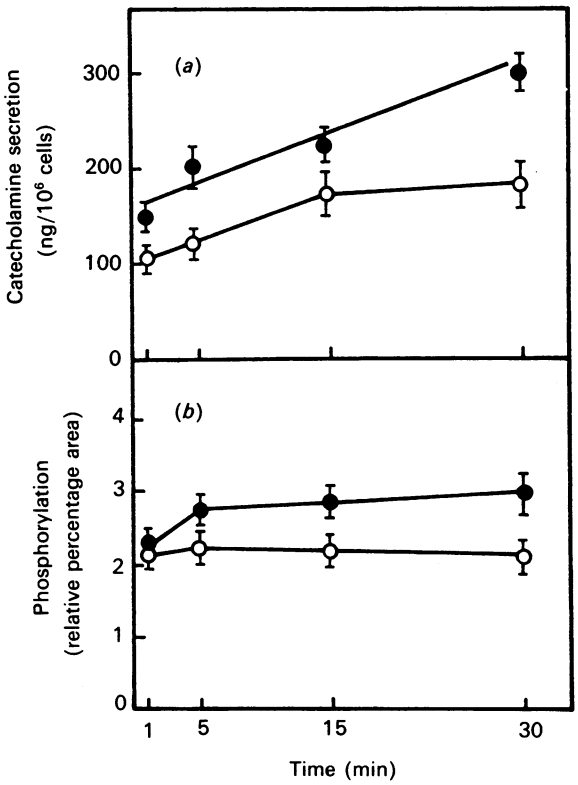

Fig. 8. Time course of catecholamine release (*a*) and ^{32}P incorporation into MLC (*b*) in K/H with 1 μM-A23187 with (○) or without 0.1 μM-PMA (●).

Results are means ± S.E.M. for four experiments.

isoelectric points obtained for adrenomedullary MLC forms were in good agreement with the values reported for MLC in other cell types (Gagelmann *et al.*, 1984; Papadopoulos & Hall, 1989). The presence of satellite bands of MLC have also been detected by two-dimensional or urea/polyacrylamide-gel electrophoresis (Tanaka *et al.*, 1985), these being considered as multi-phosphorylated forms of MLC.

Cell stimulation by high K^+ or the physiological secretagogue acetylcholine provides a well-defined time course of catecholamine secretion and a concomitant profile of MLC phosphorylation. Both processes were abolished when Ca^{2+} was absent from the stimulus-containing medium. The Ca^{2+}-channel activator Bay-K-8644, that enhances $^{45}Ca^{2+}$ uptake under K^+ stimulation in chromaffin cells (Garcia *et al.*, 1984), increased the level of ^{32}P incorporation into MLC, indicating that this protein was not saturated in its extent of phosphorylation under high K^+ stimulation alone. Moreover, the data obtained from the two-dimensional electrophoretic analysis clearly confirm the Ca^{2+}-dependent enhancement of MLC phosphorylation during stimulation, being especially evident for the more acidic polypeptide, which is probably a heavily phosphorylated form.

Since both processes, secretion and MLC phosphorylation, are decreased by Ca^{2+} deprivation, enhanced in the presence of Ca^{2+} and further increased by the Ca^{2+}-channel activator Bay-K-8644, it seems reasonable to conclude that both processes run in parallel.

The calmodulin antagonist TFP inhibits catecholamine release triggered by high K^+ or acetylcholine in cultured chromaffin cells (Kenigsberg *et al.*, 1982). This drug was able to block simultaneously the amount of ^{32}P incorporated into MLC and K^+-evoked secretion after 1 min of stimulation. This correlation was not evident at the longest time assayed (5 min) because the catecholamine secretion was developed to its full extent during the first minute, whereas the amount of ^{32}P incorporated into MLC was decreased by the effect of this drug. The TFP effect observed in MLC phosphorylation indicates the possible role of a Ca^{2+}-calmodulin-sensitive kinase in the phosphorylation stimulated by depolarization. The MLC kinase (MLCK), a Ca^{2+}-calmodulin-dependent kinase, was partially purified from bovine adrenal medulla (Kanda *et al.*, 1985). This protein regulated the '*in vitro*' actin-activated Mg^{2+}-ATPase activity of adrenomedullary myosin. On the other hand, it has been reported that ML-9, a specific inhibitor of MLCK, inhibits dopamine (3,4-dihydroxyphenethylamine) secretion induced by high K^+ in rat pheocromocytoma PC12 cells (Nagatsu *et al.*, 1987). Taken together, these results clearly suggest that MLCK might be implicated in MLC phosphorylation in chromaffin cells, playing, for this reason, an important role in the modulation of the excitation–secretion coupling process.

The phorbol ester PMA activates protein kinase C by increasing its affinity for Ca^{2+} (Castagna *et al.*, 1982). This compound enhances the adrenomedullary catecholamine secretion induced by the Ca^{2+} ionophore A23187 (Morita *et al.*, 1985), but not the release in the presence of high K^+ or carbachol. On the other hand, MLC can be phosphorylated by protein kinase C, as was demonstrated in the PMA experiments. However, the time course of this process was quite different from the secretion profile induced by depolarization or acetycholine stimulation. In digitonin-permeabilized chromaffin cells exposed to Ca^{2+} (at micromolar levels) and PMA for 30 min, the incorporation of ^{32}P in a 20 kDa/pI-5.02 protein has been reported (Lee & Holz, 1986). When these conditions were employed with intact cells, no phosphorylation of this protein was evident (Pocotte *et al.*, 1985). For these reasons it seems unlikely that protein kinase C might be implicated in MLC phosphorylation induced by secretagogues during short stimulation periods.

In platelets it has been demonstrated that MLC can be phosphorylated by both MLCK and protein kinase C (Naka *et al.*, 1983), and that these molecular events are associated with the activation and release of platelet granules. It is also remarkable that these two types of kinases induce a distinct secretion of 5-hydroxytryptamine (serotonin) in terms of both time course and extent and that they are associated with different sites of MLC phosphorylation.

The regulation of MLC activity by phosphorylation with those kinases has been also demonstrated in other cell types, such as smooth muscle (Ikebe *et al.*, 1987). In this context, our results suggest a possible dual regulation of adrenomedullary myosin by a Ca^{2+}-calmodulin kinase and protein kinase C. Whether MLCK is involved in chromaffin MLC phosphorylation induced by secretory stimuli and has a possible role in the secretion of a myosin ATPase activity (Adelstein & Eisenberg, 1980), or in other types of mechanisms involving the cytoskeleton is a question that requires further

investigation, as must the role of Ca^{2+} in the modulation of these molecular events.

We thank Dr B. A. Khaw (Cardiac Unit, Massachusetts General Hospital, Boston, MA, U.S.A.) for the gift of anti-(human heart MLC) serum. We thank Professor F. Hoffmeister for the supply of BAY-K-8644. The collaboration of M.A. Company in the preparation of cell cultures and M. Diez in the drawing of the Figures is gratefully acknowledged. This work was supported by grants from the Comision Asesora para la Investigacion Cientifica y Técnica (CAICYT 976/84; CICYT PB87-93) and the U.S.–Spanish Joint Committee (CCA 8411029). L.M.G. is a Fellow of the Fondo de Inversiones Sanitarias de la Seguridad Social.

REFERENCES

Adelstein, R. S. & Eisenberg, E. (1980) Annu. Rev. Biochem. **49**, 921–956

Almazan, G., Aunis, D., Garcia, A. G., Montiel, C., Nicolas, G. P. & Sánchez-Garcia, P. (1984) Br. J. Pharmacol. **81**, 599–610

Amy, C. M. & Kirshner, N. (1981) J. Neurochem. **36**, 847–854

Aunis, D., Guerold, B., Bader, M. F. & Ciesielski-Treska, J. (1980) Neuroscience **5**, 8261–8277

Bradford, M. M. (1976) Anal. Biochem. **96**, 248–254

Bravo, R. (1984) in Two-Dimensional Gel Electrophoresis of Proteins: a Guide to the Beginner (Celis, J. E. & Bravo, R., eds.), pp. 3–36, Academic Press, New York

Burgoyne, R. D. & Geisow, M. J. (1982) J. Neurochem. **39**, 1387–1396

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikawa, U. & Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851

Clarke, M. & Spudich, J. A. (1977) Annu. Rev. Biochem. **46**, 797–822

Côté, A., Doucet, J.-P. & Trifaró, J.-M (1986) Neuroscience **19**, 629–645

De Lorenzo, R. J. & Freedman, S. D (1978) Biochem. Biophys. Res. Commun. **80**, 183–192

Douglas, W. W. & Rubin, R. P. (1961) J. Physiol. (London) **159**, 40–47

Gagelmann, M., Ruegg, J. C. & DiSalvo, J. (1984) Biochem Biophys. Res. Commun. **120**, 933–938

Garcia, A. G., Sala, F., Reig, J. A., Viniegra, S., Frias, J., Fonteriz, R. & Gandia, L. (1984) Nature (London) **309**, 69–71

Gutierrez, L. M., Ballesta, J. J., Hidalgo, M. J., Gandia, L., Garcia, A. G. & Reig, J. A. (1988) J. Neurochem. **51**, 1023–1030

Haslam, R. J. & Linham, J. A. (1977) Biochem. Biophys. Res. Commun. **77**, 714–722

Hempstead, B. L., Hulczycki, A. & Parker, C. W. (1981) Biochem. Biophys. Res. Commun. **98**, 815–822

Hesketh, J. E., Aunis, D., Mandel, D. & Devilliers, G. (1978) Biol. Cell **33**, 199–208

Ikebe, M., Harshorne, D. J. & Elzinga, M. (1987) J. Biol. Chem. **262**, 9569–9573

Kaibuchi, K., Takai, Y., Sawamura, M., Hosshijima, M., Fujikura, T. & Nishizuka, Y. (1983) J. Biol. Chem. **258**, 6701–6704

Kanda, K., Sobue, K. & Kakiuchi, K. (1985) J. Biochem. (Tokyo) **97**, 961–964

Kenigsberg, R. L., Côté, A. & Trifaró, J. M. (1982) Neurosci. **7**, 2277–2286

Knight, D. E. & Kesteven, N. T. (1983) Proc. R. Soc. London Ser. B **218**, 177–179

Krueger, B. K., Forn, J. & Greengard, P. (1977) J. Biol. Chem. **252**, 2764–2773

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lee, S. A. & Holz, R. W. (1986) J. Biol. Chem. **261**, 17089–17098

Morita, K., Brocklehurst, K. W., Tomores, S. & Pollard, H. B. (1985) Biochem. Biophys. Res. Commun. **129**, 511–516

Nagatsu, T., Suzuki, H., Kiuchi, K., Saitoh, M. & Hidaka, H. (1987) Biochem. Biophys. Res. Commun. **143**, 1045–1048

Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) Nature (London) **306**, 490–492

Nishikawa, K., Tanaka, T. & Hidaka, H. (1980) Nature (London) **287**, 863–865

Papadopoulos, V. & Hall, P F. (1989) Cell Biol. **108**, 553–567

Pocotte, S. L., Frye, R. A., Senter, R. A., Terbush, B. R., Lee, S. A. & Holz, R. W (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 930–934

Shellenberger, M. K. & Gordon, J. H. (1971) Anal. Biochem. **39**, 356–372

Tanaka, T., Kamarazi, K. & Sobue, K. (1985) J. Biochem. (Tokyo) **97**, 1823–1826

Towbin, H., Staehelin, T. & Gordon, J. H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Trifaró, J.-M. & Ulpian, C. (1976) Neuroscience **1**, 483–488

Trifaró, J.-M., Ulpian, C. & Preiksatis, H. (1978) Experientia **34**, 1568–1571

Wise, B. C. & Costa, E. (1985) J. Neurochem. **45**, 227–234

Received 24 April 1989/6 July 1989; accepted 21 July 1989