The role of myosin in vesicle transport during bovine chromaffin cell secretion

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Bovine adrenomedullary cells in culture have been used to study the role of myosin in vesicle transport during exocytosis. Amperometric determination of calcium-dependent catecholamine release from individual digitonin-permeabilized cells treated with 3 μM wortmannin or 20 mM 2,3-butanedione monoxime (BDM) and stimulated by continuous as well as repetitive calcium pulses showed alteration of slow phases of secretion when compared with control untreated cells. The specificity of these drugs for myosin inhibition was further supported by the use of peptide-18, a potent peptide affecting myosin light-chain kinase activity. These results were supported also by studying the impact of these myosin inhibitors on chromaffin granule mobility using direct visualization by dynamic confocal microscopy. Wortmannin and BDM affect drastically vesicle transport throughout the cell cytoplasm, including the region beneath the plasma membrane. Immunocytochemical studies demonstrate the presence of myosin types II and V in the cell periphery. The capability of antibodies to myosin II in abrogating the secretory response from populations of digitonin-permeabilized cells compared with the modest effect caused by anti-myosin V suggests that myosin II plays a fundamental role in the active transport of vesicles occurring in the sub-plasmalemmal area during chromaffin cell secretory activity.

Key words: amperometry, bovine adrenomedullary cell, cytoskeleton, exocytosis.

INTRODUCTION

Adrenomedullary chromaffin cells have been widely used as a model to study exocytosis in neuroendocrine systems. In these cells, catecholamines are stored in specialized vesicles called chromaffin granules [1], and released in a calcium-dependent process involving granule translocation from the cytosol, vesicle docking and maturation, and finally the extrusion of granule soluble content by exocytosis [2]. Although much has been learned of the final molecular events involved in docking and membrane fusion since the proposition of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) hypothesis [3,4], the nature of the molecular mechanisms underlying vesicle transport and recruitment is much less understood. In this sense, the cytoskeleton has been shown to play a function in exocytosis in both neurons [5] and neuroendocrine cells [6,7]. More precisely, in chromaffin cells, it has been demonstrated that an F-actin network layer beneath the plasma membrane constitutes a cortical barrier that excludes vesicles from accessing their docking sites [7]. After stimulation, and calcium influx through voltage-dependent channels, a local and transient disruption of this network occurs [8], allowing vesicle access to the immediate sub-plasmalemmal area. In contrast with these studies demonstrating a cytoskeletal role as a passive barrier, there is little evidence for the participation of active-transport systems in neuroendocrine vesicle translocation. During chromaffin cell stimulation, by direct depolarization or physiological agonists, the regulatory subunit of myosin light chain is phosphorylated in a calcium-dependent manner proportional to the magnitude of the stimulus [9–11], and as such constitutes the basic mechanism in changing the interaction of this motor protein with actin in non-muscular systems. In addition, phosphorylation of non-muscle myosins by the specific enzyme myosin light-chain kinase (MLCK) may be essential for the role of such a protein in secretion, since inhibition of MLCK by a variety of chemicals, such as naphthalenesulphonamides (ML-9) [12,13] and wortmannin [14,15], abrogates MgATP-dependent and -independent catecholamine secretion in biochemical assays performed on populations of permeabilized chromaffin cells.

The present study has been undertaken to evaluate the function of myosin as a motor for vesicle transport in neuroendocrine cells, by using different types of agent to inhibit myosin activity: wortmannin, a potent but relatively unspecific inhibitor of MLCK [16], 2,3-butanedione monoxime (BDM), a chemical inhibiting conventional and unconventional myosin ATPase activity [17], and peptide-18, a high-affinity peptide that selectively blocks MLCK activity [18]. Our results, obtained by combining single-cell amperometrical exocytotic measurements in permeabilized chromaffin cells and the use of confocal immunofluorescence microscopy, demonstrate the involvement of this motor protein in the active transport of chromaffin granules in the immediate sub-plasmalemmal area as an early event needed for the docking and posterior exocytotic release of biogenic amines. Furthermore, the use of different antibodies against myosin forms suggests that type II myosin is especially abundant in the cell periphery and might be involved in such early exocytotic events, as has been suggested for neurons [19].

MATERIALS AND METHODS

Chromaffin cell isolation and culture

Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion, and further separated from debris and erythrocytes by centrifugation on Percoll gradients as described in [20,21]. Cells were maintained in monolayer cultures using Dulbecco's modified Eagle's medium supplemented with 10%

Abbreviations used: BDM, 2,3-butanedione monoxime; MLCK, myosin light-chain kinase; PI 3-kinase, phosphoinositide 3-kinase; ROI, region of interest.

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fetal calf serum, 10 μM cytosine arabinoside, 10 μM 5-fluoro-2′-deoxyuridine, 50 i.u./ml penicillin and 50 μg/ml streptomycin. Cells were harvested at a density of 150000 cells/cm^2 in 35 mm Petri dishes (Corning, Corning, NY, U.S.A.) and used between the second and fifth days after plating. All experiments were performed at room temperature (21–22 °C).

Amperometric determination of exocytosis from cultured chromaffin cells

To study secretory activity in individual cells, culture medium was replaced by Krebs/Hepes basal solution with the following composition: 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, 0.56 mM ascorbic acid and 15 mM Hepes. pH was adjusted to 7.4 using NaOH. Carbon-fibre electrodes insulated with polypropylene and with 11 μm-diameter tips were employed to monitor the release of the catecholamine content from individual chromaffin granules in cells under superfusion, as described elsewhere [21,22]. Electrodes were positioned in close apposition to the surface of the cells using high-precision hydraulic micromanipulation assessing cell membrane deformation using an Axiovert 135 inverted-stage microscope (Zeiss, Oberkochen, Germany) mounting Hoffman optics (Modulation Optics, Greenvale, NY, U.S.A.). Electrical connection was accomplished with mercury, and an amperometric potential of +650 mV versus an Ag/AgCl bath reference electrode was applied using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.). Electrochemical current due to catecholamine oxidation was digitized with an A/D converter (ITC-16, Instrutech Corp., Great Neck, NY, U.S.A.) and recorded at 200 μs/point using the program PulseControl [23] running on top of the graphical software Igor Pro (Wavematrics, Lake Oswego, OR, U.S.A.) on a PowerMac 7100 computer. Experiments were performed in cells stimulated by superfusion with a depolarizing high-potassium (59 mM) solution (obtained by iso-osmotically replacing NaCl with KCl), applied through a valve-controlled puffer tip commanded by the acquisition software and deposited near the studied cells. Cell treatment with wortmannin and BDM (both from Sigma, Madrid, Spain) was performed using 3 μM and 20 mM concentrations, respectively, in basal medium with 15–30 min exposures prior to cell secretion measurements.

Cell permeabilization with digitonin was performed using a 10 μM concentration superfused during 10 s in Krebs/Hepes basal medium lacking $CaCl_2$ and in the presence of 0.1 mM EGTA. Calcium-dependent secretion was evoked in permeabilized cells by cell superfusion with the same medium described for permeabilization with the addition of 0.2 mM $CaCl_2$ (0.1 mM free calcium). Cell incubations with the specific myosin inhibitor peptide-18 [18] were performed by superfusion of the cells for 30 s with 1 μM peptide-18 after cell permeabilization and prior to challenge by calcium-evoked secretion. For these experiments controls were carried out using the same protocol but in the absence of peptide.

Determination of catecholamine release from populations of detergent-permeabilized chromaffin cells

Secreted [^{3}H]noradrenaline was determined in digitonin-permeabilized cells as described previously [24]. Briefly, cells were incubated with [^{3}H]noradrenaline (1 μCi/ml) in Dulbecco's modified Eagle's medium during 4 h. Thereafter, monolayers were washed four times with Krebs/Hepes basal solution (see above). Cell permeabilization was accomplished with 10 μM digitonin in 20 mM Pipes, pH 6.8, with 140 mM monosodium glutamate, 2 mM $MgCl_2$, 2 mM MgATP and 5 mM EGTA. This incubation was carried out in the absence or presence of the different immunoglobulins. Following permeabilization, media were discarded and cells were incubated for an additional 10 min in digitonin-free medium in the presence or absence of the different antibodies or compounds assayed. Basal secretion was measured in 5 mM EGTA, whereas stimulated secretion was measured in medium containing 10 μM buffered Ca^{2+} solution. Media were collected and released catecholamines as well as the total cell content were determined by liquid scintillation counting.

Confocal microscopy studies of the cellular distribution of F-actin, and myosins II and V

Analysis of the distribution of the cytoskeletal barrier of F-actin was performed using rhodamine coupled with phalloidin as described previously [25]. Cells were fixed and permeabilized using a modification [26] of the method described by Lazarides [27]. Briefly, cells were fixed with 4 % paraformaldehyde in PBS for 20 min. Then cells were permeabilized with 0.2 % Triton X-100 in 3.6 % formaldehyde for 10 min. After washing with PBS, lipids were extracted using a series of incubations at 4 °C; 50 % acetone in water for 3 min, 100 % acetone for 5 min and finally 50 % acetone for 3 min. After extensive rinsing with PBS, cells were incubated for 15 min with a solution of 0.5 μg/ml rhodamine-phalloidin in PBS. After a 30 min wash the samples were mounted using a 80 % glycerol solution in PBS.

Labelling of myosin II and V in permeabilized and delipidated cells was performed overnight by incubating with a mouse monoclonal antiserum to non-muscular myosin II (1:200 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.) or polyclonal antibodies against squid myosin V [28] in 3 % BSA/PBS medium. After extensive washes, secondary goat anti-mouse or anti-rabbit antibodies coupled to FITC (Amersham, Little Chalfont, Bucks, U.K.) were incubated for 2 h, followed by three washes, and finally mounted as indicated above.

Fluorescence was investigated using a laser scanning confocal TCS Leica microscope. Usually eight confocal layers covering the total cell volume were obtained (1.25 μm thickness, z axis) and individual layers or projections were used to study fluorescence distribution as described elsewhere [25].

Dynamic confocal microscopic studies of chromaffin granule mobility

Cells were loaded with 4 μM quinacrine in culture medium for 10 min. After that, fluorescence emission by quinacrine trapped in the acidic vesicles was investigated using an Olympus Fluoview FV300 confocal laser system mounted in a BX-50 WI up-right microscope incorporating a 100× LUMPlan FI water-immersion objective. This system allows for z axis reconstruction (0.1 μm spatial resolution, 0.5 μm z slice) and time-lapse dynamic studies with time resolutions ranging from 0.1 s for acquisition of images of 200 pixels × 150 pixels (adequate for studying regions of a cell) to about 0.6 s for images of 400 pixels × 300 pixels (for visualization of the entire cell). Analysis of frames was performed using the public-domain program ImageJ (http://rsb.info.nih.gov/ij/) with plugins for region-of-interest (ROI) measurement, image averaging and comparison.

The Student's t test for unpaired samples or two-way ANOVA was used to establish statistical significance among experimental data. Samples were considered significantly different when $P < 0.05$. All data were expressed as means ± S.E.M. from experiments performed in a number (n) of individual cells or wells. The data presented represent experiments performed with cells from at least three different cultures.

RESULTS

Different inhibitors of myosin activity affect catecholamine secretion from individual permeabilized chromaffin cells

To study the possible role of myosin in modulating the secretory components of chromaffin cells, we decided to use two different inhibitors of myosin function: wortmannin, which has been used extensively as a potent inhibitor of MLCK [16], an enzyme that induces Ca^{2+}-dependent phosphorylation of myosin during catecholamine release [9–11], and BDM, a chemical inhibiting myosin ATPase activity [17]. Concentrations of 3 μM wortmannin and 20 mM BDM, with incubations of 30 and 15 min, respectively, affect the vesicular release of individual cells permeabilized with 10 μM digitonin and stimulated by superfusion with a solution containing 100 μM free calcium, as can be observed in the amperometric examples of Figure 1. Both agents decrease the number of vesicles fusing 5 s and more after cell depolarization, whereas the initial secretory burst remains relatively unaffected. These observations were confirmed by the integration and averaging of experiments conducted on numerous cells (15–56) in each experimental condition (Figure 1B). The characteristics of these cumulative curves representing single-cell secretory kinetics were clearly affected by these chemicals. Both wortmannin and BDM altered catecholamine release kinetics in early periods after stimulation, and produced inhibitions ranging from 55 to 70 % of the maximal response obtained in non-treated cells.

We have used these agents at concentrations similar to those employed in other cell systems [29–31], and which are thought to be relatively specific. Nevertheless, low concentrations of wortmannin have been shown to also inhibit the activity of phosphoinositide 3-kinase (PI 3-kinase) [16,31]. Peptide-18, a very potent (IC_{50}, 50 nM) and selective inhibitor of MLCK (it affects calmodulin kinase II at a 4000-fold higher concentration) has been described recently [18]. We incubated superfused permeabilized cells for short periods (30 s) with 1 μM peptide-18 before stimulation with 100 μM free calcium. In Figure 1 it can be observed that this peptide was able to inhibit the initial rate and extent of catecholamine secretion in a fashion similar to that of wortmannin, giving support to the notion of relative specificity for wortmannin and BDM when inhibiting secretion via myosin function.

These observations were further supported by experiments performed on permeabilized cells treated with these chemicals and repetitively stimulated with brief 10 s exposures to 100 μM free calcium. The initial pulse was inhibited by 20 % with wortmannin and 38 % in cells treated with BDM. The degree of inhibition was further enhanced in the subsequent pulses, as can be observed from the examples and average integral values shown in Figure 2, indicating that these chemicals affecting myosin function preferentially block slow phases of secretion and as a consequence affect vesicle recruitment from reserve pools [25].

Dynamic confocal microscopy demonstrates the impact of myosin inhibitors on vesicle mobility

To understand the possible causes of the different effects of myosin inhibitors on the secretory behaviour of chromaffin cells, we decided to study the mobility of chromaffin granules using

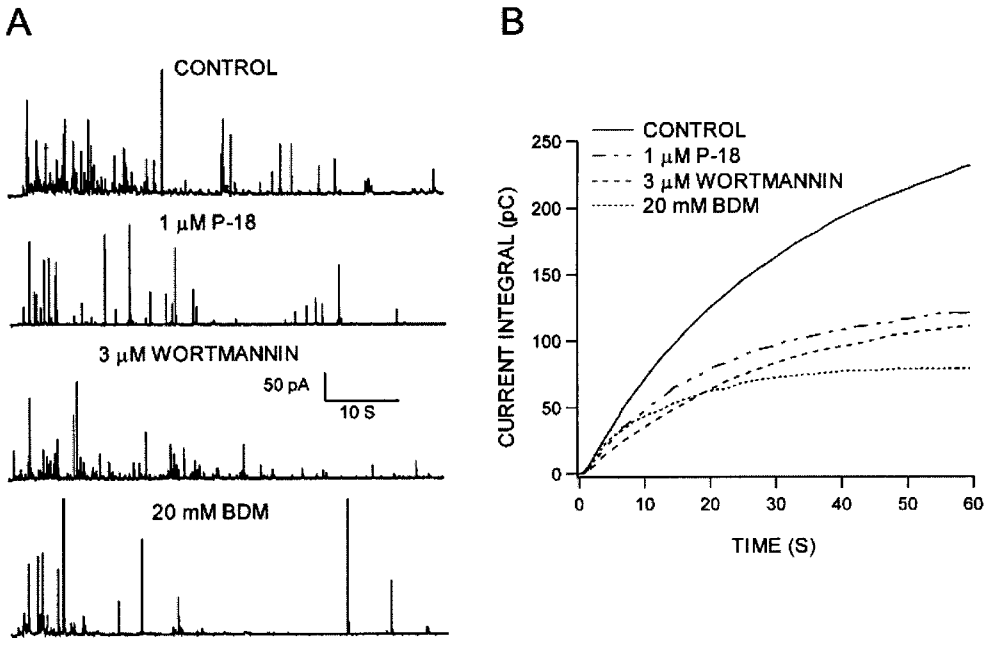

Figure 1 Secretory kinetics of individual permeabilized cells treated with myosin-activity inhibitors

In these experiments, after treatment with 3 μM wortmannin and 20 mM BDM for 30 and 15 min, respectively, secretion was elicited by cell permeabilization using a 10 s pulse of 10 μM digitonin followed by cell perfusion with Krebs/Hepes basal medium containing 100 μM free calcium. Peptide 18 (P-18) was superfused at 1 μM for 30 s in the absence of calcium prior to eliciting catecholamine release. Secretion was monitored by amperometry using 11 μm-diameter carbon-fibre electrodes in close apposition with the cell surface. (**A**) Amperometric current traces representative of experiments performed under various conditions. Traces were obtained from permeabilized control non-treated cells and wortmannin-, BDM- and peptide-18-treated cells stimulated by superfusion for 60 s with 100 μM free calcium medium. (**B**) Cumulative integrals representing averaged secretory behaviour. Conditions were as described for (**A**); after individual response integration the average curve was obtained for control ($n = 56$) and 3 μM wortmannin ($n = 37$), 20 mM BDM ($n = 15$) and 1 μM peptide-18 ($n = 16$) -treated cells. Current integral values (pC; means $\pm$ S.E.M.) at 1 min were 235 ± 22 (control), 111 ± 12 (wortmannin), 78 ± 12 (BDM) and 121 ± 14 (peptide-18).

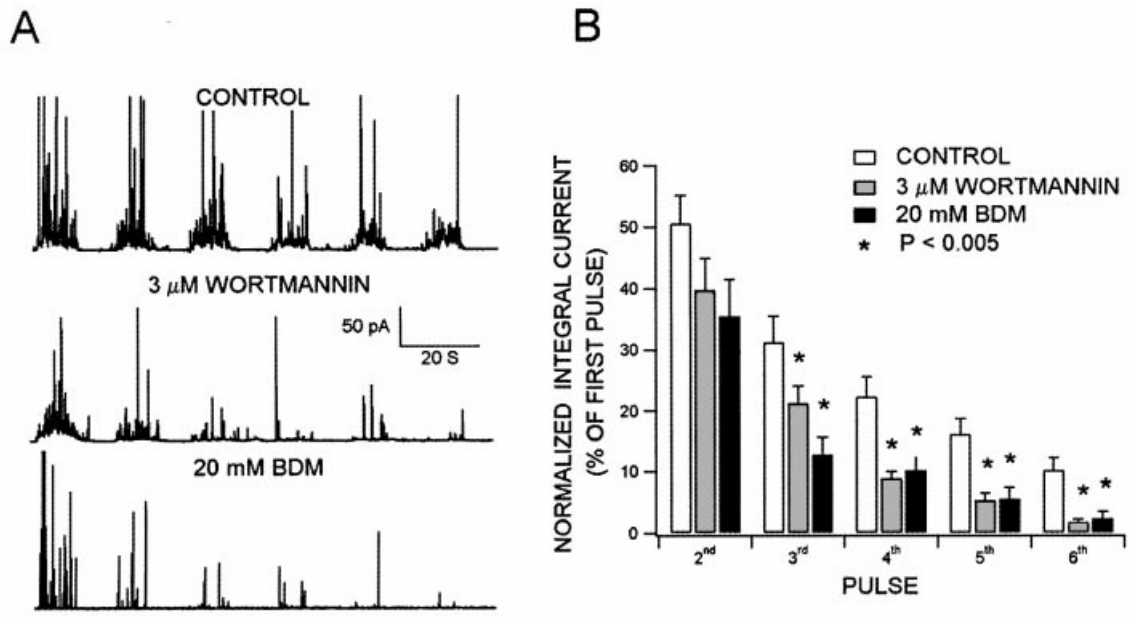

Figure 2 Wortmannin and BDM affect granule recruitment by repetitive stimulation

The capability of permeabilized cells to recruit fast or slow secretory 'pools' was studied by using six successive stimulations with 100 μM free calcium for 10 s. (**A**) Typical traces obtained from permeabilized control, 3 μM wortmannin- and 20 mM BDM-treated cells. (**B**) Rate of vesicle recruitment. The cumulative integrals were averaged for individual pulses in each experimental condition assayed and represented as a percentage of the initial pulse. Data are means $\pm$ S.E.M. from experiments performed in 38 (control non-treated), 21 (wortmannin) and 21 (BDM) cells.

laser confocal microscopy. Our aim was not to study granule trajectory throughout the cell cytoplasm [32], rather our objective was to detect clear changes in vesicle mobility after cell treatment with the conditions used in our secretory experiments and to localize the cellular zones affected by such changes in chromaffin granule motility. Chromaffin granules were labelled with 4 μM quinacrine for 10 min at 37 °C before acquiring confocal images of vesicles in the equatorial cytoplasmic sections of about 500 nm (4 μm $\times$ 2.5 μm area acquired at 10 images/s). Figure 3(A) depicts images of granules in control non-treated cells using this technique (see also compressed images in video 1, accelerated by six times; see http://www.BiochemJ.org/bj/368/bj3680405.htm). Granule movement was very noticeable, with vesicles maintaining their position in the confocal plane for no more than 2–5 s. As a consequence of this, the confocal images changed drastically over a sequence of six frames, separated by 10 s intervals (Figure 3A). This high degree of vesicle movement can also be seen in the averaged image of 60 frames taken over 1 min in which a blurry and dense representation of the multiple positions adopted by mobile granules can be observed (Figure 3B).

A further study using quinacrine fluorescence in a ROI covering the movements of 1–2 vesicles was carried out. The variation in fluorescence indicated that the granules were entering and leaving the studied ROI at intervals of 2–4 s (Figure 3E, control). These observations were made in more than 30 cells from different cultures for periods ranging from a few minutes to more than 2 h, at room temperature. These results from resting non-stimulating conditions were similar to those found in several cells exposed to a 59 mM KCl depolarizing solution, indicating that differences in vesicle motility under both sets of conditions were undetectable using this methodology. Therefore, further experiments were performed in basal medium. Our first experiments analysing the participation myosin motors for vesicle transport were performed using 3 μM wortmannin for 15 min before acquiring confocal images of the granules labelled with quinacrine. Cell treatment clearly resulted in a dramatic change in vesicle motion; confocal imaging showed that there was a marked restriction in granule movement which seemed to affect the majority of vesicles 15–20 min after initiation of the observations (see the six-times accelerated compressed images in video 2; see http://www.BiochemJ.org/bj/368/bj3680405.htm). Figure 3(C) shows a typical cytoplasmic area under these conditions; there is no noticeable change in images taken at 10 s intervals, and the averaged image of 60 confocal frames taken over 1 min is well defined and shows the position maintained by each granule during the period of observation (Figure 3D). Measurements of fluorescence intensity in ROIs containing two or three granules also indicated a marked stability, as can be seen in Figure 3(E). These results also show that there was little bleaching of the fluorescent dye at the laser intensities used. Similar results were obtained in more than 15 cells pretreated for 15 min with either 3 μM wortmannin or 20 mM BDM. These observations suggest the involvement of myosin motors in the motility of vesicles throughout the chromaffin cell cytoplasm and are consistent with studies of the immediate plasma-membrane area using total reflection microscopy [33,34].

Cortical granules are immobilized by myosin inhibitors

Although vesicle motility in the interior of the cytosol might be essential for the supply of granules to peripheral areas and therefore for the capacity of chromaffin cells to sustain the secretory response for prolonged periods of continuous or episodic stimulation, the study of granule movement in the cortical sub-plasmalemmal area is fundamental to the understanding of the effects of myosin-affecting drugs on catecholamine secretion. Confocal microscopy of this cellular area was used to

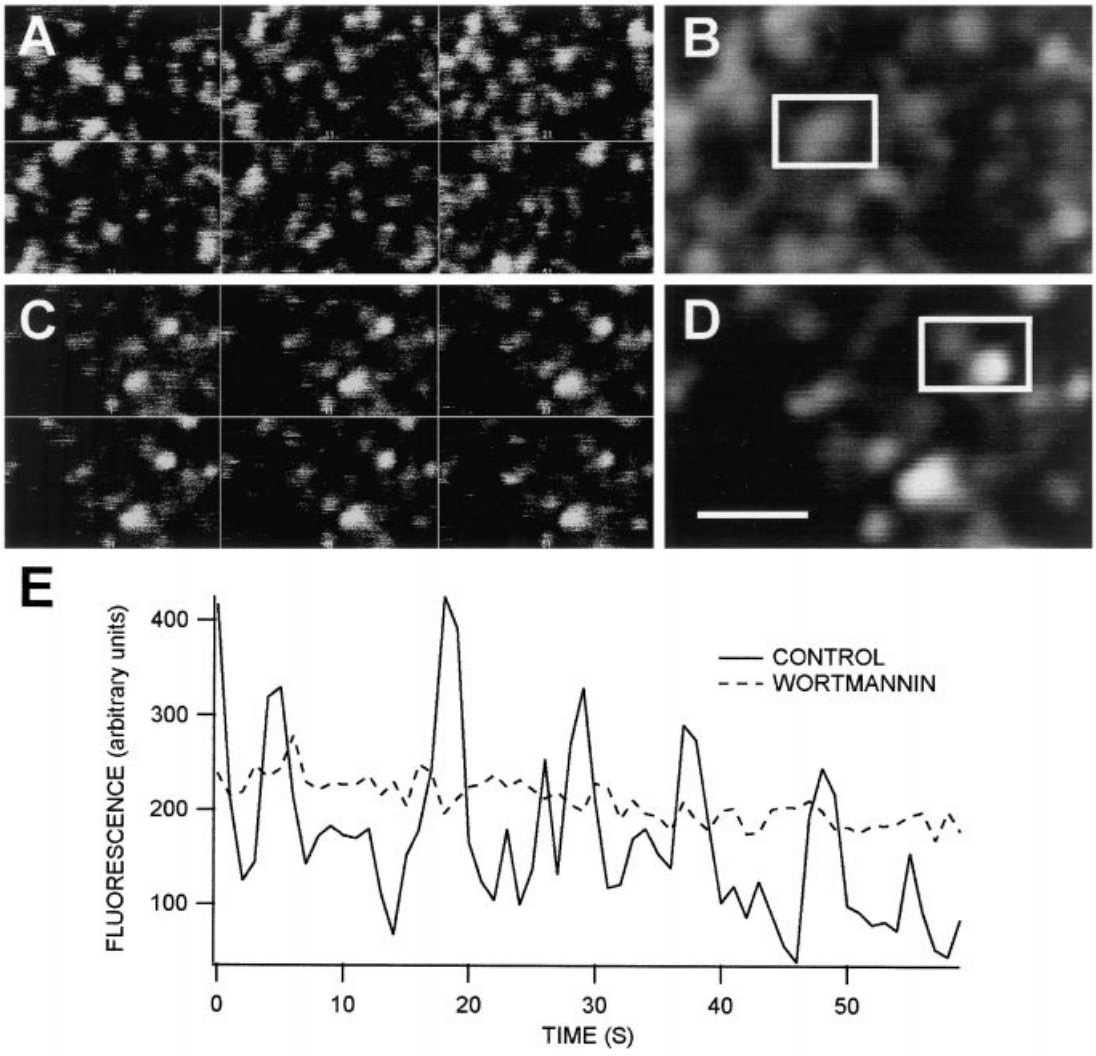

Figure 3 Vesicle mobility observed by dynamic confocal microscopy in control untreated and chromaffin cells treated with wortmannin

Chromaffin cells were incubated with 4 μM quinacrine for 10 min and granules visualized by dynamic confocal microscopy as indicated in the Materials and methods section. (**A**) Time series of confocal images taken at 10 s intervals from cytosolic vesicles in control non-treated cells. (**B**) Averaged image from 60 frames taken at 1 s intervals of granules in the control cell depicted in (**A**). (**C**) Frames obtained as in (**A**) from a cell treated with 3 μM wortmannin for 15 min. (**D**) Averaged image of vesicles in a wortmannin-treated cell. (**E**) Fluorescence changes of ROI selected in a control cell (indicated by a box in **B**) and a wortmannin-treated cell (indicated by a box in **D**). Scale bar, 1 μm.

visualize the upper portion of the cell and z axis objective movement was controlled with a precision of 0.1 μm intervals until the first image of the cell showing the most peripheral granules appeared. Chromaffin granules corresponding to control non-treated cells were then recorded at different time intervals in 17 cells from several cultures. It was observed than most of the brilliant granules (the most external and therefore immersed in the confocal plane) remained relatively immobile, suggesting a restriction of mobility by vesicle docking, whereas during the 1 min period studied other granules approached and left the confocal plane, indicating their elevated motility in the cortical zone. Most of these vesicles appeared to be moving in the direction of the z axis. A simple yet clear way to observe this behaviour is to compare the first frame of an image sequence (Figure 4A) with the averaged image of the entire period (Figure 4B), since relatively static granules are seen as brilliant and well-defined images, in contrast with the lower-intensity and blurry fluorescence characteristic of moving granules, occasional 'visitors' to the confocal plane. Upon treatment with wortmannin under the conditions described above, images of the cortical zone were remarkably static, as can be observed in the initial frame and averaged image in Figures 4(C) and 4(D). Similar images taken in 13 cells demonstrate that wortmannin treatment results in the immobilization of cortical vesicles. These results could be extended to BDM ($n = 11$), which clearly affected the movement of cortical vesicles, as seen in Figures 4(E) and 4(F).

Myosin II and V are present in the sub-plasmalemmal area of bovine chromaffin cells

What subtype of myosin could be involved in such vesicle transport? Conventional myosin II and unconventional type V have been localized in presynaptic terminals [35], and we used antibodies against these subtypes to test for their presence in fixed and permeabilized chromaffin cells. Observation under the confocal microscope revealed the specific labelling of these types

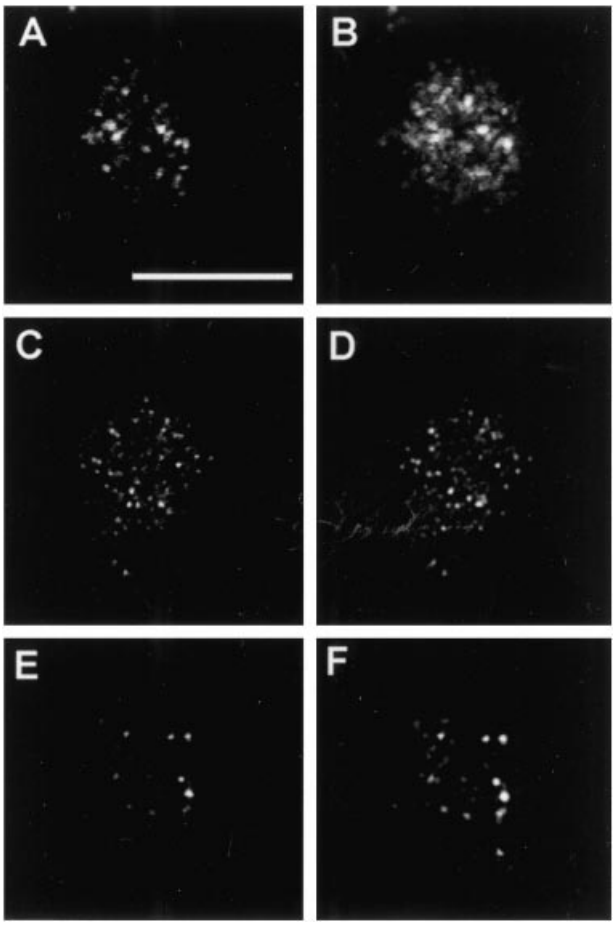

Figure 4 Mobility of cortical vesicles is affected by wortmannin and BDM

Initial frames (**A**, **C** and **E**) and average images (**B**, **D** and **F**) from time series of confocal images visualizing cortical vesicles obtained from control non-treated (**A** and **B**), 3 μM wortmannin- (15 min incubation; **C** and **D**) and BDM-treated cells (15 min incubation; **D** and **E**). Blurred average images indicated high mobility, whereas those that are well defined indicate reduced mobility. Scale bar, 10 μm.

of myosin in these neuroendocrine cells. Both forms are characterized by a punctate pattern that co-localized with F-actin filaments labelled with rhodamine-phalloidin, as observed in Figure 5. A close inspection of equatorial confocal images revealed marked differences in the distribution of these myosin classes. Myosin II was abundant in the cell periphery, where it associated clearly with the sub-plasmalemmal F-actin barrier (Figure 5A). In contrast, myosin V was concentrated in cytosolic areas, often present in a region surrounding one side of the nuclear membrane (Figure 5C), and was especially abundant in a diffuse F-actin network extending from deep zones of the cytosol to the vicinity of the peripherical F-actin barrier. Observation of the upper confocal layers corresponding to the cell periphery showed that even though myosin II is preferentially present in the sub-plasmalemmal F-actin lattice (Figure 5B), myosin V is also present (Figure 5D) in variable amounts, depending on the cell observed. Consequently, we cannot discard the possibility that both myosin types II and V could co-operate to transport vesicles in the area where the vesicles accumulate after the abrogation of secretion by myosin inhibitors.

Antibodies to myosin II and V affect secretion from permeabilized cells

To understand the possible role of these two types of myosin in sustaining the secretory response in chromaffin cells, we used the antibodies described above to test their ability in abrogating the Ca^{2+}-dependent secretory response from populations of cultured chromaffin cells, as described in previous studies [24]. The antibodies were present at a 1:200 dilution (1–2 μg/ml) in both the permeabilization buffer (incubation for 10 min) and the stimulating media in the presence or absence of 10 μM free Ca^{2+} (stimulated and basal media, respectively). Interestingly, both antibodies showed very different capabilities in inhibiting net secretion (stimulated minus basal secretion; see Figure 6), since antibodies to myosin V were inefficient at modifying the secretory response, causing only a very modest inhibition (20 % inhibition compared with the control). This effect is not statistically significant when compared with net secretion obtained in the absence of antibody treatment or in the presence of pre-immune immunoglobulins. Instead, cells treated with immunoglobulins against myosin II were clearly affected in their Ca^{2+}-dependent secretory response, since net secretion was inhibited by 64 % when compared with controls. This effect was very similar to that produced by the incubation of the cells with 20 mM BDM (Figure 6); although these values were both very different from control secretion in non-treated cells, they were not statistically different from each other. Taken together, these results indicated very different roles for both types of myosin in supporting catecholamine secretion, suggesting a preferential role for myosin II in governing the movement of vesicles in the proximity of the plasma membrane, where this form is especially abundant.

DISCUSSION

This study has been designed to evaluate the relevance of myosin activity in controlling the transport of vesicles from reserve pools needed to sustain the secretory response during prolonged or repetitive cell stimulation in bovine chromaffin cells [25]. Previous biochemical studies have demonstrated the regulation of myosin function by calcium-dependent phosphorylation of myosin light chain in parallel with secretion [9–11]. MLCK regulation by Ca^{2+}/calmodulin may play a central role in linking intracellular calcium elevation with both vesicle fusion and mobilization. Use of a variety of chemicals, such as naphthalenesulphonamides [12,13] and wortmannin [14,15], has implicated MLCK in the chromaffin cell secretory process. Our present work provides new insights into the function of myosin in secretion. We demonstrate its effect on slow secretory components, using high-resolution kinetic studies in permeabilized individual cells through the use of wortmannin, BDM and peptide-18, the latter possibly being the most specific inhibitor of MLCK activity yet synthesized [18]. Since wortmannin has been proven to affect PI 3-kinase activity, which could influence catecholamine secretion [36], the use of BDM and peptide-18 has been relevant in implicating myosin in the secretory process in chromaffin cells. In addition, we have observed that 100 nM wortmannin, which may inhibit PI 3-kinase activity, influenced neither secretion in permeabilized cells nor granule mobility in our measurements.

Our functional data show the preferential inhibition of retarded phases of secretion when continuous stimulation or multiple pulses were used to deplete mature granules. These results strengthen the notion of a fundamental role for myosin in the cargo of vesicles from reserve pools in neuroendocrine models, in agreement with results obtained in neurons [37].

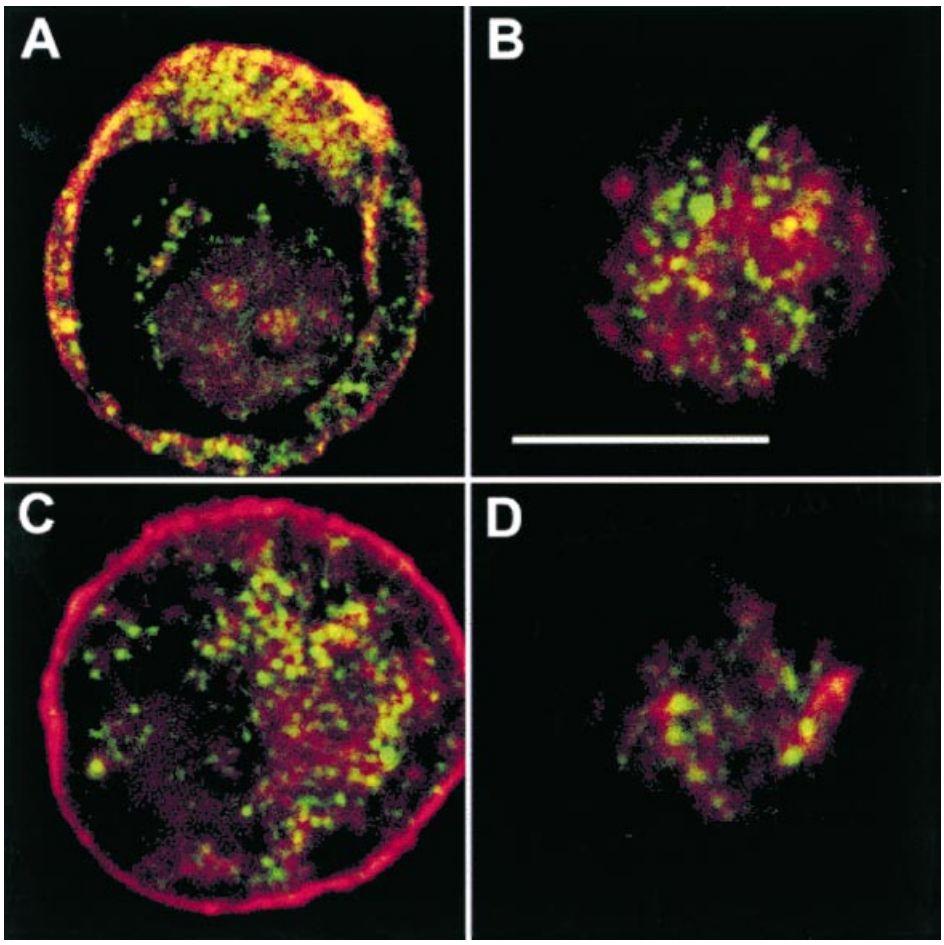

Figure 5 Distribution of myosin types II and V in bovine chromaffin cells

Fixed and permeabilized cells were incubated with antibodies against cardiac myosin II (**A** and **B**) and squid brain myosin V (**C** and **D**) at 1 : 200 dilutions. After incubation and washing of secondary antibodies labelled with FITC, the F-actin cytoskeleton was visualized using rhodamine coupled with phalloidin. Fluorescence distribution in equatorial sections (**A** and **C**) and upper layers was investigated using confocal microscopy. Scale bar, 10 μm.

In recent years a number of studies using total internal reflection microscopy have provided us with a detailed description of granule motion in the proximity of the plasma membrane [33,34,38,39]. These groups used either weak bases that accumulate in the interior of acidic vesicles (such as Acridine Orange or quinacrine, used here) or green fluorescent protein constructs with vesicular proteins to reach similar conclusions regarding the restriction of vesicle movement linked to vesicle tethering or docking. Here the use of confocal microscopy provides a global vision of this aspect of vesicle mobility throughout the cytoplasm, from deep regions where granules are being synthesized to peripheral areas where they accumulate to constitute reserve pools for sustaining secretion. The vast majority of particles labelled were spherical, with diameters varying from 150 to 400 nm (about 300 nm on average, in agreement with granule size), and in the control, non-treated cells rarely stayed within the confocal plane (estimated in 500 nm) for more than a few seconds (ROI analysis of individual granules showed average periods of 3–4 s). This dynamic behaviour changed upon treatment of the cells with myosin inhibitors, which drastically reduce vesicle motility, causing the majority of granules to remain in the confocal plane throughout the period of study (in the order of minutes). This reduction in granule motility throughout the whole cytoplasm suggests that myosin-dependent transport systems play a role in catecholamine secretion and would explain the marked effect of these agents in the functional amperometrical studies presented here. This analysis is in agreement with studies of the sub-plasmalemmal region using total internal reflection microscopy [33,34].

What might be the myosin class involved in such a function? Previous studies have shown labelling of abundant myosin beneath the plasma membrane of chromaffin cells [11,15], which has been recognized here as type II conventional myosin associated with the F-actin network. In addition, specific labelling of chromaffin cells with an antibody against myosin V from squid brain cytosol [28] shows that this subtype is present and abundant in deeper areas of the cytosol; the situation is similar in neurons, where both myosin II and myosin V have been localized in the synaptic terminals [19,35]. Clearly, distribution and labelling aspects suggest the involvement of myosin V in transport of vesicles or other organelles from the limits of the endoplasmic reticulum to the cell periphery (see Figure 5C), and the co-operation of both types of myosin in accomplishing vesicle transport through the exocytic pathway could not be ruled out. Nevertheless, the preferential abrogation of Ca^{2+}-dependent catecholamine release in permeabilized cells using

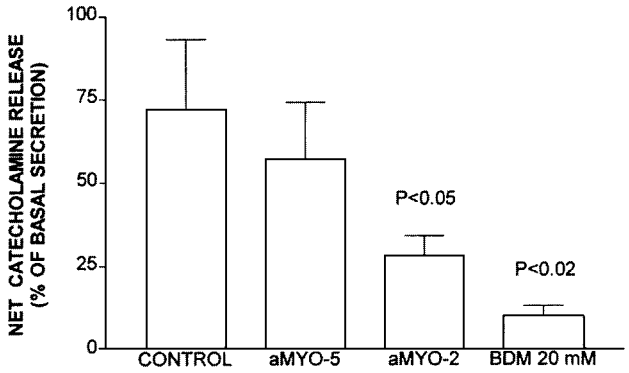

Figure 6 Anti-myosin II antibodies affect the release of catecholamines from populations of digitonin-permeabilized chromaffin cells

Cultured chromaffin cells (200 000 cells/1 cm-diameter well) were first incubated for 3 h with [^{3}H]noradrenaline and then washed extensively as described in the Materials and methods section. Cells were permeabilized with 10 μM digitonin for 10 min in the presence or absence of 1:200 dilutions of antibodies against myosin II (aMYO-2) and V (aMYO-5). Secretion was then elicited for 10 min in a medium lacking or containing 10 μM free Ca^{2+}, again in the presence or absence of the antibodies assayed. In addition, some wells were treated with 20 mM BDM for 15 min prior to cell permeabilization. Net catecholamine secretion was calculated as the difference between the presence and absence of Ca^{2+} for every experimental condition. Secretion is expressed as a percentage of catecholamine released in the absence of added calcium in non-treated cells (basal secretion). Incubation with pre-immune antibodies did not affect the control response. *P* values were calculated using the Student's *t* test for paired samples (12 wells for experimental condition).

antibodies against myosin II and the relatively minor effects displayed by anti-myosin V suggest the pivotal role of the type II form in participating in or controlling some of the molecular events taking place during exocytosis of catecholamines in neuroendocrine chromaffin cells. Further research using molecular tools designed to interfere specifically with myosin classes is clearly needed to establish the precise nature of myosin molecules involved in different stages of vesicle transport and exocytosis in this neuroendocrine cellular model.

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