Monensin Turns on Microtubule-Associated Translocation of Secretory Granules in Cultured Rat Atrial Myocytes

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We have studied the effect of monensin on microtubule-associated translocation of atrial secretory granules in 5–7-day-old cultures of atrial myocytes from adult rats. Atrial granules and microtubules were localized by immunofluorescent microscopy of myocytes double-labeled with primary antibodies against atrial natriuretic polypeptide (ANP) and α-tubulin. In control myocytes, fluorescence due to atrial granules was predominantly localized to the perinuclear region containing the Golgi complex. After exposure for 30 minutes to monensin (0.5–5.0 μM), myocytes transiently contained conspicuous linear arrays of atrial granules associated with cytoplasmic microtubules. Thereafter, ANP fluorescence accumulated in subsarcolemmal foci at the cell periphery, while perinuclear ANP fluorescence faded. The monensin-induced redistribution of atrial granules was observable in both serum-containing and serum-free media and was unaffected by reducing external Ca²⁺ to low values, by inhibition of sarcoplasmic reticulum Ca²⁺ release with ryanodine, or by both. The redistribution was prevented by pretreatment with nocodazole, which fragmented microtubules and scattered Golgi complexes and the associated atrial granules throughout the cytoplasm. Radioimmunoassay showed that monensin seemingly decreased the rate of ANP secretion into the medium from 0.15 to 0.11 fmol/(hr myocyte). These results suggest that monensin turns on microtubule-associated translocation of atrial granules from the perinuclear areas to the cell periphery by modifying the interaction between microtubules and atrial granules. Monensin also promotes movement of atrial granules along the microtubules but does not accelerate the release of ANP. (Circulation Research 1988;62:1159–1170)

The cytoplasm of myocytes in mammalian atria is the locus of characteristic vesicles (atrial granules) that contain atrial natriuretic polypeptides (ANP) that have potent natriuretic, diuretic, and vascular smooth muscle relaxant activities. Although these peptides are important for the maintenance of blood pressure, blood volume, and cellular ionic composition, their processing, intracellular transport, and secretion by atrial myocytes from adult animals are poorly understood.

In frog atria, monensin, a Na⁺-selective ionophore, raises the cytoplasmic ionized Na⁺ and Ca²⁺ concentrations by an effect on plasmalemmal Na⁺ and K⁺ transport. Monensin also abolishes the proton gradient across the membranes of lysosomes, secretory vesicles, and those parts of the Golgi complex normally acidified by vanadate-insensitive proton pumps in their limiting membranes. These effects of monensin have been used to study processing, sorting, intracellular transport, and exocytosis of secreted proteins.

A third effect of monensin—that of stimulating the translocation of secretory granules from the nuclear poles to the cell periphery—has been suggested for cultures of chromaffin cells. Intracellular translocation of secretory granules seems to be necessary for ANP secretion, but little is known about this step in the secretory process in atrial myocytes. Therefore, we investigated the details of atrial granule translocation in myocytes cultured from atria of adult rats, in the presence and absence of monensin. These studies have shown that monensin stimulates microtubule-associated transport of atrial granules from the perinuclear regions to the cell periphery but does not accelerate the release of ANP into the medium.

Materials and Methods

Isolation and Culture of Atrial Myocytes

Sprague-Dawley rats (300–350 g, either sex, chosen because they were large) were anesthetized with ether. Both atria were excised and rinsed with low calcium medium (LCM; see below) containing heparin (50 units/ml). The atria were cut into eight pieces. Calcium-tolerant, dispersed atrial myocytes were made by a minor modification of the method of Isenberg and Klockner for ventricular myocytes. After rinsing, the pieces of atria were transferred to a petri dish (diameter 60 mm) for enzymatic dissociation in LCM containing collagenase (1–2 mg/ml; Cooper Biomedical, Malvern, Pennsylvania) and bovine serum albumin (1%). The pieces were gently agitated in this solution at about 35°C on a shaking thermostated water bath set at one stroke per second. The dissociating solution was continuously oxygenated with 100% O₂ saturated with water vapor. After 60 minutes of incubation, the original solution was replaced by an identical solution con-
taining fresh enzyme, and the atrial tissues were incubated for an additional 20 minutes. The cells were then released from the pieces of undissociated atrium by gentle pipetting. Two additional 20-minute periods in dissociating medium, each followed by gentle pipetting, resulted in nearly complete dissociation at the end of the total 2-hour exposure to collagenase. Released cells were collected by centrifugation, resuspended in an oxygenated modified KB medium (see below), and stored in this medium at 22–24°C for at least 45 minutes. After incubation in the modified KB medium, isolated atrial myocytes were transferred to culture dishes (diameter 60 mm) containing Medium 199 (GIBCO, Grand Island, New York) plus 10% fetal bovine serum (FBS; GIBCO) and the antibiotics penicillin (100,000 IU/l) and streptomycin (0.1 g/l). The cells were allowed to settle in an incubator for 4–5 hours at 37°C. After this preculture period, the unattached cells (myocytes with reduced nonmyocyte contamination) were replated at a density of 0.2–0.7 × 10⁶ cells/cm² onto 35-mm culture dishes or 13-mm glass coverslips coated with 40 µg/ml collagen type IV (Sigma Chemical, St. Louis, Missouri). Immunofluorescence microscopy was done on cells attached to glass coverslips; electron microscopy and immunocytochemistry (see legend of Figure 10 in detail) were performed on cells attached to culture dishes. Cytosine-1-β-D-arabinofuranoside (ARA-C; 5–10 µg/ml) was added to the culture medium on days 0 and 3 of culture to inhibit the surviving cells were supplemented with fresh culture medium. ARA-C was removed at least 1 day before use of the cultured cells for experiments. Atrial myocytes had spread fully over the substrate after 4–5 days of culture, as reported by Moses and Claycomb. All experiments were done within 5–7 days of culture.

Solutions. The composition of LCM was (mM) NaCl 120, KCl 5.4, MgSO₄ 5, Na pyruvate 5, glucose 20, and taurine 20. The LCM was buffered to pH 7.3 with HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid). CaCl₂ was added to give a nominal calcium concentration of 10 µM. The modified KB medium contained (mM) taurine 20, glutamic acid 70, KCl 25, KH₂PO₄ 10, glucose 11, HEPES 10, and EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid] 0.5, pH 7.3. The inorganic ion composition of GIBCO Medium 199 was (mM) Ca 1.8, K 5.4, Na 150, Mg 0.8, Cl 130, HCO₃ 26, and phosphate 1.0; the medium was buffered to pH 7.3 with 10 mM HEPES.

Immunofluorescence Microscopy

As described by Reggio et al., atrial cells grown on glass coverslips were fixed for 10 minutes at 22–24°C with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) [composition (g/l): NaCl 8.5, Na₂HPO₄·7H₂O 2.14, and NaH₂PO₄ 0.32]. Fixed cells were washed with PBS, incubated with 50 mM NH₄Cl for 5 minutes, permeabilized by 0.1% Triton X-100 for 3 minutes, and incubated with PBS containing 0.2% gelatin (PBS-gelatin) for 15 minutes. The cells were stained with primary antibody for 1 hour in a water vapor-saturated atmosphere and then stained for 1 hour with a secondary antibody. The primary antibody was a 1:500 dilution of rabbit α-human ANP antiserum (Peninsula Laboratories, Belmont, California). We have confirmed that this antiserum stains atrial granules specifically by immunoelectron microscopy with the method of Tokuyasu. The secondary antibody was a 1:100 dilution of FITC- or TRITC-conjugated goat anti-rabbit immunoglobulin (Southern Biotechnology Associates, Birmingham, Alabama). Antibodies were diluted with PBS-gelatin. The controls for antibody staining consisted of substituting pre-immune rabbit serum for the primary antibody. For labeling cells doubly with anti-ANP and anti-tubulin antibodies, the cells were incubated sequentially with rabbit anti-ANP antibody, TRITC-conjugated goat anti-rabbit Ig, mouse monoclonal anti-α-tubulin antibody (Amersham, Arlington Heights, Illinois), and FITC-conjugated sheep anti-mouse Ig (Amersham). The mouse monoclonal anti-α-tubulin antibody and the sheep anti-mouse immunoglobulin were diluted 1:500 and 1:20, respectively, with PBS-gelatin. The Golgi complex was localized by staining the permeabilized cells with 150 µg/ml TRITC-conjugated wheat germ agglutinin (WGA; E-Y Laboratories, San Mateo, California). WGA, which binds to N-acetyl-glucosamine, is used as a marker for the Golgi complex.

Specimens were viewed with an Olympus BH-12 microscope (Scientific Supply Co., Schiller Park, Illinois) equipped with epifluorescence optics and filters for FITC and TRITC. The specimens were photographed with the ×100 oil-immersion objective using Kodak Ektachrome 400 film.

Experiments With Monensin and Nocodazole

Monensin (final concentration 0.5 or 5.0 µM) and nocodazole (10 µM) (both from Sigma) were dissolved in ethanol and dimethyl sulfoxide (DMSO), respectively, to obtain final solvent concentrations of <0.1%. This solvent concentration produced no detectable changes in morphology or immunostaining. Cells were incubated with these agents for designated times in the presence of the 10% serum culture medium and then prepared for immunofluorescent or electron microscopy. Controls were incubated without monensin or nocodazole in the presence of <0.1% concentration of ethanol or DMSO, as appropriate.

Two additional series of experiments were done to determine the effects of monensin at different external Ca²⁺ concentrations and in nominally Ca²⁺-free solutions containing different concentrations of ryanodine. In the first series, four dishes of the same culture of atrial myocytes were incubated for 30 minutes in
Hanks’ solution containing 5 μM monensin and external Ca²⁺ concentrations of 1.26 mM, 0.1 mM, 0 mM (nominally Ca²⁺-free; no added Ca²⁺), and 0 mM Ca²⁺ plus 0.5 mM EGTA, respectively. In the second series, four dishes of the same culture of atrial myocytes were incubated for 30 minutes in Hanks’ solution containing 5 μM monensin and 0 mM Ca²⁺ (nominally Ca²⁺-free) plus 0, 100, 10, and 1 μM, respectively, of ryanodine. At the end of the 30 minutes, the cells were immunostained and examined for fluorescence of secondary antibody against ANP.

Determination of ANP Concentration in Culture Medium

Synthetic rat α-atrial natriuretic factor (α-ANF, molecular weight 3062.92 for the standard curve) and antibody to rat α-ANF (RAS 8798) were obtained from Peninsula Laboratories, Belmont, California. Cross-reactivity of the antibody (RAS 8798) with rat α-ANF was 100%. ¹²⁵I-α-ANF was prepared using a modification of the chloramine-T method or was obtained from Amersham; the assay buffer was 0.1 M sodium phosphate at pH 7.4. One hundred microliters of standard or unknown was incubated in duplicate with antibody for 24 hours at 4°C, followed by the addition of 4,000 cpm of ¹²⁵I-tracer and incubation for an additional 24 hours. In assays using tracer made in our laboratory, bound and free hormones were separated by the addition of 50 μl 1.5% bovine γ-globulin and 500 μl 20% polyethylene glycol followed by centrifugation at 4,000 rpm for 30 minutes; when iodinated peptide from Amersham was used, separation was achieved by the addition of 1 ml charcoal suspension (0.08% Dextran T70, 0.16% bovine γ-globulin, and 0.42% charcoal) in 0.1 M sodium phosphate buffer. Nonspecific binding averaged 4.2%. Sensitivity of this assay was 1.9 pg/tube; the 50% displacement was 18 pg. Culture medium containing no ANF did not demonstrate significant displacement, and measurements of serial dilutions (1:25 to 1:200) of cell culture supernatants with and without monensin yielded curves that were parallel to the standard curve.

Results

Distribution of Antibody to ANP in Atrial Myocytes

When examined under phase contrast, cultured atrial myocytes were seen to assume different shapes. Most of the cells contained either one or two nuclei. Dense granules (presumed to be mitochondria or lysosomes) were present in the cytoplasm. Myocytes often extended several cell processes by which they sometimes made contact with other myocytes (Figure 1). Electron micrographs of thin sections showed that the Golgi complex was confined to the perinuclear region (Figure 2). The Golgi consisted of four or five cisterns and was associated with secretory granules and other vesicles. Fluorescence microscopy of WGA-stained cells suggested an apparently interconnected perinuclear arrangement of the Golgi (Figure 3). Staining with fluorescent secondary antibody against rabbit α-human atrial natriuretic antiserum (ANP antibody staining) revealed a tendency of ANP-containing storage vesicles (atrial granules) to accumulate circumferentially around the nuclei (Figure 4). The number of granules per cell was variable.

Effects of Monensin on Distribution of Atrial Granules

Exposure of atrial myocytes for 30 minutes to monensin at concentrations of 5 μM (Figures 5A, 6B, and 7B) or 0.5 μM (Figure 5B) caused atrial granules to rearrange themselves in linear arrays. In favorable orientations and focus, individual granules could be clearly identified in these arrays (Figures 5B and 6B). Incubation in 0.5 μM monensin for 3 hours (Figures 5C and 5D) or for 7 hours (Figure 5E) resulted in accumulation of ANP-associated fluorescence in the cell periphery (including the cell processes), while ANP-associated perinuclear fluorescence diminished markedly. Omission of serum from the medium in which cells were incubated for 3 hours with 0.5 μM monensin produced a monensin-induced redistribution of ANP antibody staining (Figure 5D) indistinguishable from that in presence of serum (Figure 5C). Control cells incubated without monensin for 7 hours (Figure 5F) never showed a redistribution of ANP.

Microtubules and Monensin-Induced Redistribution of Atrial Granules

Multiple published studies on secretory cells have suggested that microtubules are implicated in the intracellular translocation of vesicles containing concentrated secretory proteins (or their precursors). We, therefore, examined the effect of treating atrial myocytes for 30 minutes with 5 μM monensin on the relation between the distributions of microtubules and atrial granules, by both electron microscopy (Figure 8) and immunofluorescence microscopy (Figure 9). Figure 9E illustrates the arrangement of microtubules under control conditions (without monensin or nocodazole). The microtubules were densely packed throughout the perinuclear and peripheral cytoplasm,
FIGURE 1. Phase-contrast micrograph of rat atrial myocyte cultured for 7 days. Calibration bar, 50 μm.

FIGURE 2. Electron micrograph of cultured atrial myocyte showing perinuclear area containing Golgi apparatus and ANP granules. Calibration bar, 0.5 μm.

FIGURE 3. Fluorescence micrograph of cultured atrial myocyte stained by tetramethylrhodamine isothiocyanate-conjugated wheat germ agglutinin. Golgi complex is visualized as darkened areas around nuclei. Calibration bar, 10 μm.

FIGURE 4. Immunofluorescence micrograph of cultured atrial myocytes stained by anti-α-ANP antibody. Note tendency of secretory granules to accumulate around nuclei. Calibration bar, 10 μm.
FIGURE 5. Effects of monensin on distribution of ANP granules in cultured atrial myocytes stained with anti-α-ANP antibody. Atrial myocytes were treated for (a) 30 minutes with 5 μM monensin, (b) 30 minutes with 0.5 μM monensin, (c and d) 3 hours with 0.5 μM monensin, or (e) 7 hours with 0.5 μM monensin. Thirty minutes' incubation with monensin caused redistribution of ANP granules in linear arrays (a) in which individual granules were sometimes identified (b, arrows). Longer incubation with monensin caused accumulation of ANP granules in cell periphery (c, d, and e) regardless of presence (c) or absence (d) of serum in culture medium. Incubation with 0.01% ethanol for 7 hours did not affect distribution of ANP granules in cells (f). Calibration bar, 10 μm.
including the cell processes; the perinuclear region was most densely stained. Incubation for 30 minutes in 5 μM monensin had no discernible effect on microtubular organization, as reported for cultured skeletal myoblasts and myotubes by Tassin et al.9 The double immunostaining technique for tubulin (Figures 6A and 7A) and ANP (Figures 6B and 7B) was used to show that granules are arrayed linearly along microtubules in the monensin-treated cells. Linear structures labeled with fluorescein and corresponding structures labeled with rhodamine were traced onto separate sheets of tracing paper, and then the two tracings were superimposed by orienting them with respect to appropriate “landmarks.” In this way, we confirmed that most of the arrays labeled with ANP antibodies completely overlapped the corresponding labeled microtubules.

Electron microscopy (Figure 8A) confirmed the co-distribution of microtubules and atrial granules in cells treated with monensin for 30 minutes, a distribution that corresponds to that already demonstrated by immunofluorescence microscopy (Figures 6A, 6B, 7A, and 7B). In addition, the higher resolution of the electron micrographs showed a 10–20 nm gap between the microtubule and the associated atrial granular membrane (Figure 8B). Dilatation of some of the Golgi cisterns, a classical effect of monensin,4 was also seen (Figure 8C). Prolonged incubation with monensin for more than 3 hours caused morphological changes in some of the atrial granules (Figure 8D). The ultrastructure of cultured rat atrial cells not exposed to monensin (Figure 8E) was as previously reported in detail by Moses and Claycomb.13

**Nocodazole Experiments**

To confirm that microtubules are involved in the process by which ANP-containing granules become redistributed in linear arrays, the cells pretreated with 10 μM nocodazole for 2 hours were further incubated for 30 minutes with both 5 μM monensin and 10 μM nocodazole. The pretreatment procedure produced a random distribution of ANP-associated fluorescence in the cells (Figure 9A). In addition to individual granules, the cytoplasm of the atrial myocytes contained both large patches and smaller foci of fluorescence. In general, the large patches tended to be localized closer to the nuclei than to the smaller foci. Some of the large patches remained attached to the nuclei. Electron microscopic examination confirmed that the large patch-es and small foci correspond to atrial granules associated with fragmented Golgi complexes and aggregates of several granules, respectively. Most cells pretreated for 2 hours with nocodazole failed to respond to subsequent exposure to monensin by redistribution of granules (Figure 9B). Controls pretreated for 2 hours with DMSO only (omitting nocodazole) responded uniformly to subsequent monensin treatment by a redistribution of atrial granules similar to that of Figure 5A. Double immunostaining for ANP and tubulin confirmed that incubation with 10 μM nocodazole for 2.5 hours reduced microtubules to short fragments (Figure 9C) and dispersed the foci of ANP-associated fluorescence (Figures 9D). Short fragments of microtubules were not eliminated, even when the nocodazole concentration was raised to 20 and 30 μM. By contrast, microtubules were absent in fibroblastlike cells (a contaminating cell type) after exposure to 10 μM nocodazole. In control, atrial myocytes incubated for 2.5 hours with 0.1% DMSO, the morphology and distribution of microtubules (Figure 9E), and ANP (Figure 9F) were normal. As previously reported for isolated ventricular myocytes,20 microtubule-disrupting drugs had little effect on the shape of cultured atrial cells.

**Effects of External Ca²⁺ Concentration and Ryanodine**

To test whether the transient microtubule-associated translocation of atrial granules produced by monensin is dependent on external Ca²⁺ concentration, we examined the effect of monensin on atrial myocytes from the same culture incubated for 30 minutes in four dishes containing Hanks’ solution, monensin (5 μM), and Ca²⁺ concentrations of 1.26 mM, 0.1 mM, nominally 0 mM, and 0 mM plus 0.5 mM EGTA, respectively. In each of these solutions, immunofluorescence microscopy showed the same linear arrays of atrial granules on cytoplasmic microtubules characteristic of the monensin effect. To test whether the monensin-induced, microtubule-associated translocation of atrial granules depended on release of Ca²⁺ into the cytoplasm from the sarcoplasmic reticulum, we examined a similar series of four dishes incubated for 30 minutes in nominally Ca²⁺-free Hanks’ solution containing 5 μM monensin and ryanodine at concentrations of 0, 1, 10, and 100 μM, respectively. Immunofluorescence microscopy of each of these cultures again showed characteristic linear arrays of atrial granules on microtubules. These results indicate that the monensin-induced translocation of granules on cytoplasmic microtubules did not require a transplasmalemmal influx of extracellular Ca²⁺ or the release of Ca²⁺ from the sarcoplasmic reticulum.

**Effects of Monensin on ANP Secretion**

The above morphological studies showed that monensin stimulated microtubule-associated translocation of atrial granules from the perinuclear areas to the cell periphery, where the secretion-containing granules accumulated beneath the sarcolemma, suggesting that monensin did not accelerate the release of ANP. We, therefore, examined the rate of ANP secretion
FIGURE 8. Electron micrographs of atrial myocytes treated with monensin (a, b, c, and d) and control treated with solvent (ethanol) only (e). a, b, and c: Cells treated for 30 minutes with 5 μM monensin, showing close relation between microtubules and atrial granules (a, b); existence of 10-20-nm gap between granule membrane and microtubule (b); and dilatation of Golgi and Golgi-associated vesicles (c). d: Myocyte exposed to 0.5 μM monensin for 3 hours, showing profiles of some vesicles with reduced secretory content, limited to their core (arrows); caveolae in left lower corner of field indicate proximity to cell surface. e: Control incubated for 60 minutes with ethanol (0.1%) shows only normal perinuclear distribution and appearance of atrial granules. Calibration bars, (a) 0.2 μm; (b) 0.2 μm; (c) 1 μm; (d) 1 μm; and (e) 2 μm.

by radioimmunoassay in presence and absence of monensin.

Figure 10 is a graph showing the time courses of the concentrations of immunoreactive ANP (in picomoles per milliliter) in the culture medium under control conditions and in the presence of 0.5 μM monensin in 0.01% ethanol. The data for picomoles of immunoreactive polypeptide accumulated per milliliter of culture medium from 30 to 300 minutes were fitted to straight lines by the method of least-squares. For the controls, this procedure yielded a line, \( y = 0.068x + 1.8 \) pmol immunoreactive ANP accumulated/ml, whose mean slope (±SEM) [0.068 ± 0.002 pmol immunoreactive ANP accumulated/(ml·min)] and extrapolated intercept on the ordinate \( (1.78 ± 0.29) \) were both significantly different from 0 \( (p = 0.0001) \). The corresponding line for the monensin-treated cultured cells was \( y = 0.051x + 1.2 \) pmol immunoreactive ANP accumulated/ml; its slope \[0.051 ± 0.001 \mathrm{pmol \ immunoreactive \ ANP \ accumulated/(ml·min)}\] and intercept \( (1.16 ± 0.27) \) were also significantly different from 0 \( (p = 0.0015 \text{ and } 0.0001, \text{ respectively}) \). Furthermore, the slopes of the two lines differed significantly \( (p = 0.01) \). Under control conditions, the rate of ANP secretion between 30 and 300 minutes was 4.07 pmol/ml·hr or 0.15 fmol/hr·myocyte: in monensin-treated cultures, the rate slowed to 3.04 pmol/ml·hr or 0.11 fmol/hr·myocyte.

Discussion

Our observations demonstrate for the first time a clear-cut association between cytoplasmic microtubules and atrial granules during translocation of the granules from the perinuclear region to the cell periphery. Thus, the cytoplasmic microtubules provide the tracks along which atrial granules move in atrial myocytes as they do in other secretory cell types. Conditions for the demonstration of microtubule-associated translocation of granules were especially favorable in monensin-treated atrial myocytes during the massive peripheral movement of secretion-containing vesicles induced by this ionophore. The transient acceleration of granule transport during this period greatly increased the probability of observing granules associated with microtubules, an association that was too inconspicuous to detect reliably under the conditions of steady-state ANP secretion in absence of monensin. The abolition of the monensin-induced translocation of granules by depolymerizing microtubules with nocodazole supports the conclusion that intact microtubules are necessary for the directed peripheral movement of atrial granules.

Our morphological studies suggest that monensin modulates the interactions between cytoplasmic microtubules and atrial granules, thereby stimulating movement of the granules to the cell periphery. Although not visualized by electron microscopy of positively stained thin sections as used in this study, the structural connections (cross-bridges) between cytoplasmic microtubules and the membranes of vesicles (connections that might be involved in organelle transport) have been observed electron-microscopically in axoplasm after quick-freezing and deep-etching, and after negative staining, as well as in extruded
Figure 9. Effects of nocodazole on distribution of ANP granules and microtubules in cultured atrial myocytes. Incubation for 2 hours with 10 μM nocodazole produced dispersal of ANP-associated fluorescence, which appears as large and small foci in cytoplasm (a). Dispersed distribution was not detectably changed by subsequent incubation for 30 minutes with 5 μM monensin plus 10 μM nocodazole (b). Atrial myocytes treated for 2.5 hours with 10 μM nocodazole in 0.1% DMSO, then doubly stained by anti-α-tubulin antibody (c) and anti-α-ANP antibody (d). Myocytes for controls (e and f) were prepared similarly to c and d, except that only 0.1% DMSO without nocodazole was used. Partial destruction of microtubules (c) by nocodazole treatment led to dispersion of ANP granules (d) in cytoplasm. DMSO had no effect on distributions of microtubules (e) and ANP granules (f). Calibration bars, 10 μm.
squid axoplasm subjected to focal cold block. Our observation that monensin-induced association of atrial granules with microtubules was insensitive to reduction of extracellular Ca\(^{2+}\) concentration to the very low levels prevailing in 0.5 mM EGTA, to inhibition of Ca\(^{2+}\) release from the sarcoplasmic reticulum with ryanodine, or to a combination of these two experimental manipulations suggests that association between atrial granular membranes and cytoplasmic microtubules as well as the resulting granule movement along the microtubules was not critically dependent on transplasmalemmal Ca\(^{2+}\) influx or on sarcoplasmic reticulum Ca\(^{2+}\) release under the conditions of our experiments. In this connection, it is noteworthy that our primary cultures of rat atrial myocytes contain a significant basal level of ryanodine-insensitive ANP secretion in low Ca\(^{2+}\) solutions and that the phorbol ester-stimulated increase in this basal rate requires reduction of extracellular Ca\(^{2+}\) concentration to the significant basal level of ryanodine-insensitive ANP secretion observed in our experiments. Our measurements on primary cultures of atrial myocytes from adult rats (references 27 and 28; the present study; and H. Iida, W. M. Barron, and E. Page, unpublished work) like those of Sylvestre et al\(^{22}\) on atrial myocytes cultured from neonatal rat hearts, indicate that secretion is sustained at a constant rate for prolonged periods, even when the absolute rates of (con- stant) secretion differ substantially. More information on the regulation of the various steps in both secretory pathways is required to explain these observations.

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**FIGURE 10.** Atria from 11 adult rats were dissociated as described in "Materials and Methods." Cells were plated onto 25 dishes (diameter 35 mm) coated with type IV collagen and cultured for 7 days. The cells were washed three times with culture medium only and then incubated for specified intervals with 2 ml culture medium supplemented with 10% FCS and containing either 0.5 \(\mu\)M monensin in 0.01% ethanol (C) or 0.01% ethanol only (>). At end of specified interval, samples of medium were collected and immediately frozen at \(-70^\circ\) C for subsequent radioimmunoassay. Each point in graph represents mean of duplicate calculations from one culture dish. Cell number per dish (5.96 \(\times 10^4\) cells) was determined on cells fixed and stained with 1% toluidine blue by projecting a 2 \(\times\) 2 \(\times\) 2 mm area of dish at \(\times 50\) onto screen of Nikon model 6C-2 Profile Projector. Percentage of total cell population made up of atrial myocytes (89% ANP-granule-containing myocytes) was determined by staining cells with ANP antibody and tetramethylrhodamine isothiocyanate-conjugated secondary antibody as described in "Materials and Methods." Cell viability was examined by Trypan blue exclusion. Viability was greater than 96%, even after incubation for 5 hours with 0.5 \(\mu\)M monensin in 0.01% ethanol or in 0.01% ethanol only.

adult atria, longer incubation with the ionophore apparently decreased the rate of ANP secretion into the medium and caused granules to accumulate in the cell periphery. At the very least, this result suggests that monensin does not accelerate the release of ANP. Partial inhibition of ANP secretion by monensin might be due to its interference with protein synthesis, protein processing, or the recycling of the secretory vesicle membranes. It is also likely that such partial inhibition is related to the morphological changes in atrial granules seen in cells exposed to monensin for a long time (Figure 8D). Thibault et al\(^{23}\) have reported that monensin fails to increase ANP secretion by atrial myocytes cultured from hearts of neonatal rats, a result consistent with our measurements on atrial myocytes from hearts of adult rats.

It is probable that atrial myocytes, like other protein-secreting cells, can secrete aprotinin peptides by both the regulated pathway (microtubule-associated cytoplasmic translocation of concentrated prohormone stored in atrial granules and secreted by exocytosis) and the constitutive pathway (diffusion of unconcentrated prohormone through the cytoplasm from the Golgi to the plasma membrane). The present study focuses on the regulated pathway. The effects of monensin (and other experimental perturbations) need not affect both pathways to the same degree or in the same way, and the accelerated microtubule-associated translocation of atrial granules caused by monensin need not necessarily lead to acceleration of the terminal step or steps involved in exocytotic extrusion of the secretory product. For these reasons, it is not surprising that we found no straightforward relation between the rate of ANP secretion (the sum of the secretory rates by the regulated and constitutive pathways) and the time course of atrial granule translocation.

Studies on insulin secretion by pancreatic \(\beta\)-cells show that transport of secretory granules toward the periphery of the cell along microtubules is not necessary for the immediate release of hormone from the cells but is essential for constant, sustained secretion. Our measurements on primary cultures of atrial myocytes from adult rats (references 27 and 28; the present study; and H. Iida, W. M. Barron, and E. Page, unpublished work) like those of Sylvestre et al\(^{22}\) on atrial myocytes cultured from neonatal rat hearts, indicate that secretion is sustained at a constant rate for prolonged periods, even when the absolute rates of (constant) secretion differ substantially. More information on the regulation of the various steps in both secretory pathways is required to explain these observations.
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