Stimulation of Catecholamine Secretion from Cultured Chromaffin Cells by an Ionophore-mediated Rise in Intracellular Sodium

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ABSTRACT The significance of intracellular Na^+ concentration in catecholamine secretion of cultured bovine adrenal chromaffin cells was investigated using the monovalent carboxylic ionophore monensin. This ionophore, which is known to mediate a one-for-one exchange of intracellular K^+ for extracellular Na^+, induces a slow, prolonged release of catecholamines which, at 6 h, amounts of 75–90% of the total catecholamines; carbachol induces a rapid pulse of catecholamine secretion of 25–35%. Although secretory granule numbers appear to be qualitatively reduced after carbachol, multiple carbachol, or Ba^{2+} stimulation, overall granule distribution remains similar to that in untreated cells. Monensin-stimulated catecholamine release requires extracellular Na^+ but not Ca^{2+} whereas carbachol-stimulated catecholamine release requires extracellular Ca^{2+} and is partially dependent on extracellular Na^+. Despite its high selectivity for monovalent ions, monensin is considerably more effective in promoting catecholamine secretion than the divalent ionophores, A23187 and ionomycin, which mediate a more direct entry of extracellular Ca^{2+} into the cell. We propose that the monensin-stimulated increase in intracellular Na^+ levels causes an increase in the availability of intracellular Ca^{2+} which, in turn, stimulates exocytosis. This hypothesis is supported by the comparable stimulation of catecholamine release by ouabain which inhibits the outwardly directed Na^+ pump and thus permits intracellular Na^+ to accumulate. The relative magnitudes of the secretion elicited by monensin, carbachol, and the calcium ionophores, are most consistent with the hypothesis that, under normal physiological conditions, Na^+ acts by decreasing the propensity of Ca^{2+}-sequestering sites to bind the Ca^{2+} that enters the cell as a result of acetylcholine stimulation.

The key role of Ca^{2+} in stimulus-secretion coupling in secretory cells was first recognized by Douglas and Rubin (1). The subsequent availability of divalent cation ionophores, e.g. A23187, lasalocid (X-537A), ionomycin, prompted an extensive series of investigations of the ability of these agents to promote cellular secretion by admitting extracellular Ca^{2+} into cells (2–11).

A number of recent studies indicate that a rise in the cytoplasmic Na^+ concentration also stimulates the secretory activity of cells (12–15). The Na^+ may dislodge a small fraction of the relatively large store of intracellularly sequestered Ca^{2+} so that it becomes available to the presumptive intracellular Ca^{2+} receptors, calmodulin or troponin C (16). Alternately, increased intracellular Na^+ may interfere with the ability of the intracellular Ca^{2+}-sequestering mechanisms to intercept Ca^{2+} that is brought into the cytosol by secretagogue-initiated processes. Ouabain, which alters the egress of intracellular Na^+ by a specific inhibition of the Na^+-K^+-ATPase (Na^+ pump), elevates intracellular Na^+ levels and thereby stimulates secretion of catecholamines, acetylcholine, and insulin (17–20).

During the past decade, significant data have accumulated which imply that catecholamine secretion can be stimulated by ionophore-mediated increases in intracellular Ca^{2+} and Na^+ activity. In intact animals, the broad spectrum ionophore lasalocid produces certain cardiovascular effects by virtue of its ability to transport Ca^{2+} and thereby raise intracellular Ca^{2+}

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activity (21). Additionally, partial inhibition of these cardiovascular effects by β-adrenergic blockers such as propanalol, implies that lasalocid also causes the release of catecholamines. A subsequent study demonstrated that other carboxylic ionophores, even those which are incapable of transporting Ca^{2+}, duplicate most of the cardiovascular effects of lasalocid (22). In fact, monensin, a Na^+-selective ionophore, evokes similar, but even more pronounced, cardiovascular effects (23). Monensin-induced cardiovascular effects are also partially inhibited by β-adrenergic blockers (24) which suggests that monensin, like lasalocid, causes catecholamine release. This background demonstrates the existence of a manipulable Na^+ effect in catecholamine secretion which in turn suggests that catecholamine secreting cells are an appropriate system for investigating the role of Na^+ in secretion.

The present study was designed to test the hypothesis that the elevation of intracellular Na^+ by the Na^+-selective carboxylic ionophore monensin (or by ouabain) will stimulate catecholamine secretion in cultured adrenal chromaffin cells. To demonstrate that these cells respond in a manner comparable to the intact gland, the cultured cells were exposed to agents that stimulate catecholamine secretion (high K^+, Ba^{2+}, and carbachol). Since we presume that Na^+ acts by increasing the effectiveness of intracellular Ca^{2+}, we also compared the effects of monensin to carboxylic ionophores that are able to transport Ca^{2+} directly across biological membranes, namely A23187 and ionomycin.

MATERIALS AND METHODS

Chemicals and Supplies

All reagents were analytical grade unless otherwise specified and double distilled water was used for all solutions. A23187 and monensin were gifts from Eli Lilly and Company (Indianapolis, IN). Ionomycin was a gift from E.R. Squibb and Sons (Princeton, NJ). Carbamylcholine (carbachol), tris (hydroxymethyl) aminomethane, ouabain, hexamethonium, dimethylsulfoxide, 5-fluorodeoxyuridine, gentamicin sulfate and collagenase (Type I) were obtained from Sigma Chemical Company (St. Louis, MO). Fungizone, penicillin-streptomycin, fetal calf serum (FCS), and Dulbecco's modified Eagle's media (DME) were purchased from Gibco (Grand Island Biological Co., Grand Island, NY). New England Nuclear supplied Aquasol II, [^{3}H]norepinephrine and [^{3}H]*S*-adenosylmethionine. Nylon mesh with a pore size of 250 μm was obtained from Small Parts, Inc. (Miami, FL). Percoll was purchased from Pharmacia Fine Chemical (Uppsala, Sweden).

Preparation of Chromaffin Cell Cultures

Isolated bovine adrenal medullary chromaffin cells were prepared and maintained in culture using modifications of the methods described by Fenwick et al. (25) and Kilpatrick et al. (5). After perfusion of fresh adrenal glands with 0.1% collagenase (5), the medullas were removed and minced. The medullary tissue was subjected to three successive 0.1% collagenase digestions at 37°C in a shaking water bath under 95% O_2/5% CO_2. The fluid from these digestions was pooled and filtered through nylon mesh to remove undigested material from the isolated medullary cells. To remove contaminating erythrocytes and cellular debris, the filtrate was centrifuged at 48,000 *g* through 50% Percoll. After isolation, the cells were pelleted, washed, and resuspended in DME containing FCS (10%) and antibiotics (0.5% penicillin-streptomycin; 0.5% gentamicin; 1.0% fungizone); 5-fluorodeoxyuridine (5 μM) was added to inhibit fibroblast growth. The cells were plated in 24-well cluster plates (19 cm^2/well) at a concentration of 3 to 4 × 10^5 cells/well and maintained at 37°C in 5% CO_2/95% air for both experimental and morphological studies. Before conducting experiments, cells were held in culture for at least 72 h, an interval we found requisite for the cells to become fully responsive to carbachol stimulation.

Agents Used to Stimulate Catecholamine Secretion

The characteristics of catecholamine secretion in our culture system were determined by exposing the cells to the acetylcholine analog carbachol (10^{-4} M) and the cations K^+ (56 mM) and Ba^{2+} (5 mM), substances known to trigger the release of catecholamines from the medulla of intact adrenal glands (26, 27). The carbachol concentration selected produced the maximal release of catecholamines from cultured cells. These responses were used as a standard against which to compare results of experimental manipulations in which intracellular concentrations of specific ions were artificially elevated.

Because of our interest in the role of Na^+ in secretion, we chose to use the monovalent-ion-selective ionophore monensin (28) which elevates the intracellular Na^+ concentration directly by promoting a one-for-one exchange of extracellular Na^+ for intracellular K^+ down the prevailing transcellular gradients. The intracellular Na^+ concentration was also elevated by blocking the Na^+-K^+-ATPase with ouabain (10^{-6} M) (29). Hexamethonium (10^{-3} M), a cholinergic blocker (30) was used to block acetylcholine receptor-mediated secretion. Gross intracellular Ca^{2+} concentration was elevated by exposing the cells to the calcium-selective ionophores, ionomycin (31) and A23187 (32). Another mechanism for raising intracellular Ca^{2+} is to raise the extracellular K^+ concentration which mimics the action of secretagogues in depolarizing the cell membrane and causing an influx of extracellular Ca^{2+} (27, 33). Cells were exposed to concentrations of K^+ over the range of 5.6 mM to 110 mM. The cultured cells were exposed to monensin, A23187, and ionomycin over a concentration range of 10^{-10} to 10^{-5} M. Monensin, ionomycin, and ouabain were dissolved in ethanol, whereas A23187 was dissolved in dimethylsulfoxide (DMSO). The concentration of ethanol or DMSO in the incubation medium was always 0.5% at which concentration normal basal levels of catecholamine secretion were maintained.

The medium was assayed for the cytoplasmic enzyme, lactate dehydrogenase (LDH) (34) to establish that the cells were not being damaged by the various experimental manipulations. The presence of LDH in the incubation medium would indicate injury to the plasma membrane.

All experiments were conducted either in a standard or one of various modified Locke's solutions, the compositions of which are listed below. For experiments with ionophores, carbachol or ouabain, standard Locke's solution (156 mM NaCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, and 10 mM glucose) was used. High K^+ Locke's solution contained 56 mM K^+ of which 53 was KCl and 3 mM was K_2HPO_4 and KH_2PO_4. In this solution NaCl was reduced by an equivalent amount to 105.4 mM. In the series of experiments where a range of concentrations from 5.6 mM to 110 mM K^+ were used, KCl was increased from 2.6 mM to 107 mM, K_2HPO_4 and KH_2PO_4 were maintained at 3 mM, and NaCl was reduced from 156 mM to 51.6 mM. In the Na^+-free and 10 mM Na^+ Locke's solutions all the NaCl and all but 10 mM NaCl respectively of the standard Locke's solution was replaced by sucrose. Ca^{2+}-free Locke's solution and Ba^{2+} Locke's solution were prepared by omitting the 2.2 mM $CaCl_2$ or replacing it with 5 mM $BaCl_2$ respectively. All solutions were adjusted to pH 7.4 by the addition of NaOH or, in the case of the Na^+-free solution, tris (hydroxymethyl) aminomethane.

Measurement of Catecholamine Release

Catecholamine secretion was determined by measuring the release of [^{3}H] norepinephrine from labeled cells (5). These results were corroborated on occasion by parallel experiments in which endogenous catecholamines were assayed by the radioenzymic *O*-methyl-transferase procedure (35). Kilpatrick et al. (5) have established the validity of using cultured adrenal medulla cells, prelabeled with [^{3}H]norepinephrine, to study secretion. They observed that the secretion of radioactive ([^{3}H]norepinephrine) and endogenous catecholamines is identical when expressed as the percent of total catecholamine content. Unless otherwise indicated each culture well represents one data point. Secretion was measured as the amount of catecholamine released and presented as the percent of the total catecholamine content, plotted as the mean ± SEM of experiments performed in triplicate. For each experiment, the total catecholamine content was determined by adding the amount of catecholamine released during the experiment to the amount of catecholamine remaining in the cells at the end of the experiment. Each experiment was repeated two to three times, with similar results each time. For the sake of clarity, the results of representative experimental series are presented.

In preparation for measurement of [^{3}H]catecholamine release, the cells were labeled by incubation for 18 h at 37°C in 0.5 ml of DME containing 0.01% ascorbic acid and 2 μCi of 1-[^{3}H]norepinephrine (5–15 Ci/mM). Paper chromatography verified that [^{3}H]norepinephrine did indeed persist as such within the incubated cells 24 h after labeling. Before each experiment, ^{3}H-labeled cells were washed twice with 1 ml DME for 15 min per wash, and then quickly rinsed twice with Locke's medium. Experiments were initiated by replacing the Locke's rinse with 0.5 ml Locke's solution with or without secretory agents and the cells then incubated at 37°C. At a given time point, all of the incubation medium was removed and added to scintillation vials for counting. To determine the [^{3}H] norepinephrine remaining in the attached cells, 0.5 ml of 0.1 N $HClO_4$ was added and the cell lysate counted.

In experiments where multiple drug treatments were carried out on the same

individual well, the incubation medium was removed after a given time interval and replaced with fresh (sometimes different) incubation medium. The media were analyzed for catecholamine content and at the end of the experiment the amount of catecholamine remaining in the cells was determined.

Electron Microscopy

The cells were fixed and embedded in the multi-well cluster plates in which they were cultured. The incubation medium (control or experimental solutions) was removed, the cells washed twice in isotonic salt solution (Locke's medium), and then fixed at room temperature in 2.5% glutaraldehyde (vol/vol) with Locke's medium as buffer. Fixation time varied from 1 h to several days. If fixation was for more than an hour, the cells were stored at 4°C. After fixation, the cells were rinsed in Locke's solution and postfixed at room temperature in 1–2% OsO_4 (vol/vol) in Locke's medium. The cells were then processed and embedded in Epon 812 according to the technique of Brinkley et al. (36) which maintains cell/substrate associations intact. Ultrathin sections (80–100 nm) were cut with a diamond knife on a Sorvall MT2-B ultramicrotome (DuPont Instruments, Newtown, CT), stained with 2% aqueous uranyl acetate and lead citrate (37), and examined on a Phillips 300 transmission electron microscope.

RESULTS

Characterization of Chromaffin Cell Cultures

Immediately after plating, cultured adrenal chromaffin cells are rounded. However, within 24 h, the cells firmly attach to the bottom surface of the culture wells and begin to flatten and spread over the substrate by extending long pseudopodlike processes. By 48 to 72 h, the cells flatten and spread to form a confluent layer on the culture surface. Before confluence, cultures are poorly responsive to secretagogues. Because of this consideration, all experiments were conducted after 72 h of primary culture. Even though superficial examination indicates uniform distribution over the culture surface, ultrastructural examination at 72 h frequently revealed clumps of 5 to 7 cells, often in association with a few cortical cells. This same organization was observed in the primary cultures of Kilpatrick et al. (5). Our cultures contain both types of chromaffin cells (Fig. 1) previously described from the medulla of intact adrenal glands (38). Both cell types contain typical, membrane-bound chromaffin granules, and differ only in the electron-density of these granules.

When cultured chromaffin cells are exposed to carbachol, K^+, or Ba^{2+} for 20 min, the pharmacological responses are similar to those of the intact gland (26, 27, and Fig. 2). High K^+ stimulates a 15–17% release of catecholamines, whereas Ba^{2+} stimulates a 50–55% release. Carbachol, which transiently stimulates the acetylcholine receptor on the external surface of the plasma membrane, induces a 25–30% release of catecholamines. To determine if acetylcholine receptors can be repetitively stimulated by carbachol, we attempted to "reactivate" the receptors with additional 20 min incubations with carbachol after 1-min incubations in carbachol-free medium (Fig. 3). Three successive sequences of reactivation and carbachol addition released an additional 15% catecholamine; the total release being ~45–50%. A fourth carbachol stimulation yielded no additional catecholamine release. Although secretory granule numbers appear to be qualitatively reduced after a single carbachol stimulation, the granule distribution appears similar to that in untreated cells and granules are not selectively cleared from the cytoplasm immediately adjacent to the plasma membrane (Fig. 4). In both Ba^{2+} (Fig. 5) and multiple carbachol stimulated cells (Fig. 6), the granule population is greatly reduced and, as in the untreated and carbachol-treated cells, the granules do not appear consistently localized in any particular region of the cell.

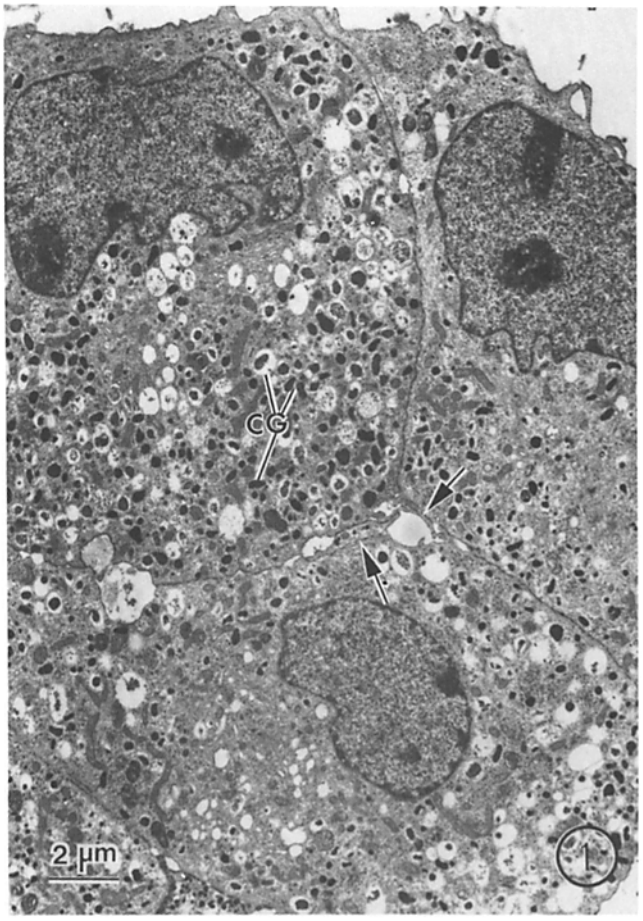


FIGURE 1 Ultrastructure of adrenal chromaffin cells after 72 h in culture. Membrane-bound catecholamine containing chromaffin granules (CG) appear to be uniformly distributed throughout the cytoplasm of these cells. Adjacent chromaffin cells are closely apposed and their cell surface membranes interdigitate (arrows). × 4,950.

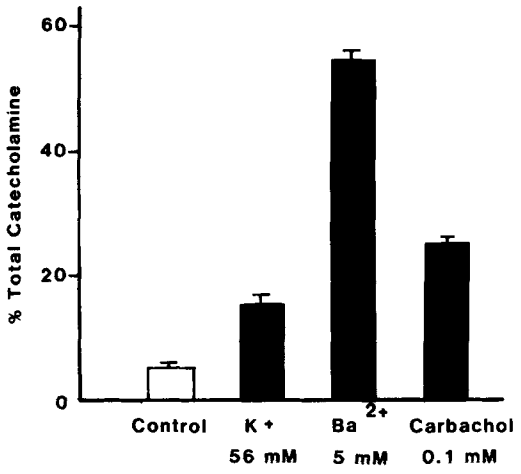


FIGURE 2 Histogram comparing catecholamine release from cultured chromaffin cells in the presence of K^+ (56 mM), Ba^{2+} (5 mM), and carbachol (0.1 mM). All exposures were for a 20-min period. Secretion, in this and the following figures, was measured by determining the catecholamine content of the media after stimulation (see Materials and Methods). These values are expressed as a percent of the total catecholamine content of cells before stimulation. Values are mean ± SEM for three culture wells.

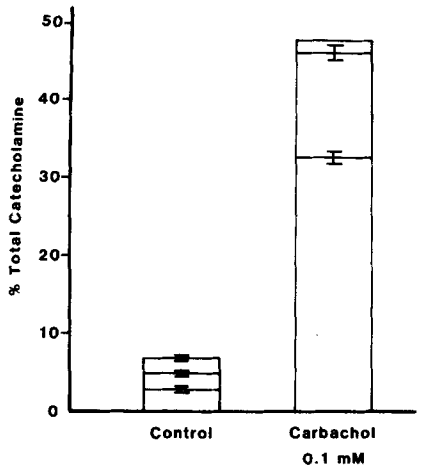
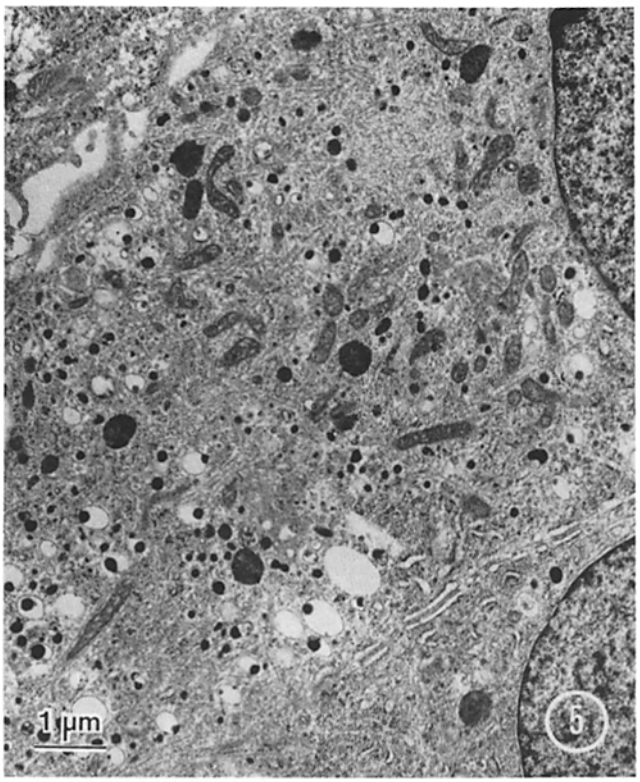


FIGURE 3 The effect of multiple carbachol treatments on cultured chromaffin cells. Cells were repetitively dosed with carbachol (10^{-4} M) for 20-min intervals. After each interval, the media and three subsequent washes were pooled. The catecholamine content for each time interval are presented as one column to illustrate the total catecholamine released with multiple carbachol stimulations. The three successive control values indicate the percentage catecholamine content released during experimental manipulation. The response to carbachol diminished at each consecutive dose and a fourth treatment yielded no further release. Values are mean ± SEM for three culture wells. An error bar is not shown for the third carbachol stimulation as the line thickness exceeds the length of the bar.



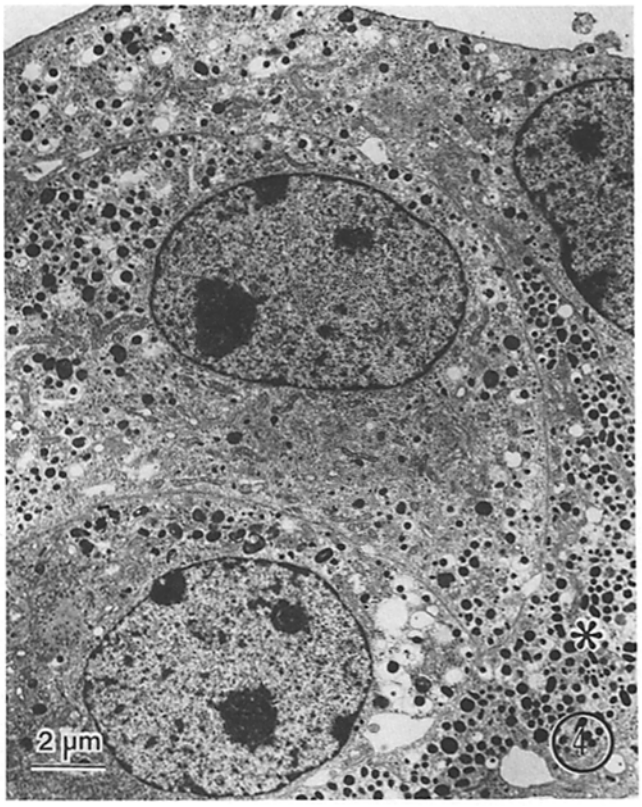
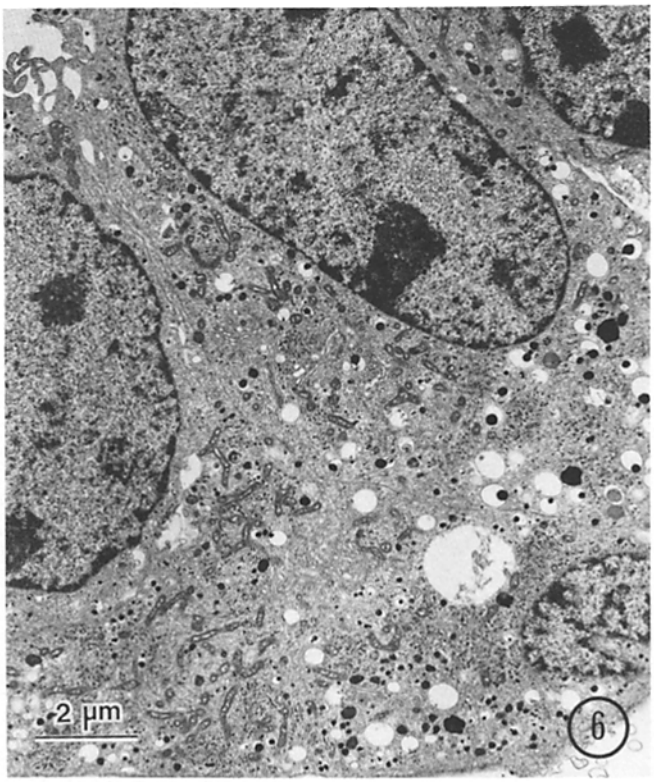


FIGURE 4 Cultured chromaffin cells after a single application of carbachol (10^{-4} M). Although secretory granule numbers appear to be qualitatively reduced, the overall granule distribution is unaltered. Occasionally a cell is observed that does not appear to respond to carbachol stimulation (*). × 4,950.



FIGURES 5 and 6 In both Ba^{2+} (Fig. 5) and multiple-carbachol (Fig. 6) stimulated cells, the chromaffin granule population is greatly reduced but granule distribution is relatively unchanged; i.e. granules are not selectively cleared from any region of the cytoplasm. Fig. 5, × 9,300; Fig. 6, × 6,750.

Monensin-stimulated Secretion

To evaluate the role of Na^+ in secretion, we used the sodium-ionophore monensin to elevate the intracellular Na^+ concentration. The results obtained in the presence of monensin were then compared to the results obtained with carbachol stimulation, since it mimics a typical physiological response. The time courses of basal, carbachol-stimulated, and monensin-stimulated catecholamine release from cultured chromaffin cells are shown in Fig. 7. Within 6–10 min of exposure, the maximum response to carbachol stimulation occurs and 30–35% of the total catecholamine are secreted. The cells respond to monensin (10^{-6} M) stimulation more slowly, but an equivalent release of catecholamines occurs in 2.5 h. The response to monensin is linear for 6 h by which time the cells have released 75–90% of their total catecholamine content compared to ~30% for a single exposure to carbachol. Conversely, continued exposure to carbachol for 6 h did not cause a significant increase in catecholamine secretion beyond that observed during the first 10 min of exposure. Pretreating cultures with hexamethonium (10^{-3} M) blocked carbachol-stimulated release of catecholamines but had no effect on monensin-triggered secretion (Fig. 8). The difference in the rate and magnitude of the secretory response to these two drugs, as well as the differing response after pretreatment with hexamethonium, indicates that carbachol and monensin are acting by different mechanisms.

The lipophilic nature of ionophores (39) suggests that monensin cannot be readily removed from the membranes of treated cells by simple washing. To test this hypothesis, cultured chromaffin cells were pretreated with monensin for either 20, 90, or 150 min, washed, and catecholamine release measured over the remainder of the 5-h time period. For comparison, cells were exposed to monensin for the entire 5-h interval as well. Regardless of whether the monensin exposure was 20 min or 5 h, the percent of the total catecholamine released and the kinetics of release were the same (data not shown).

To demonstrate the ionic requirements for the release of catecholamines by carbachol and monensin, we tested the ability of these drugs to stimulate catecholamine release in media deficient in Na^+ or Ca^{2+} (Fig. 9). In low sodium (10 mM), carbachol-induced secretion is 55% of control and monensin-induced secretion is 70% of its control. When extracellular Na^+ is totally replaced by an osmotic equivalent of sucrose, carbachol-induced secretion remains 55% while monensin-induced secretion is reduced to 14%. When Na^+ is replaced with Li^+, to maintain the same ionic strength, similar results are obtained. In Ca^{2+}-free medium, monensin-induced secretion is 80% of control while carbachol-stimulated secretion is only 2%. These results indicate that the response to carbachol is dependent on extracellular Ca^{2+} and partially dependent on extracellular Na^+ while the response to monensin is relatively independent of extracellular Ca^{2+} and dependent on extracellular Na^+.

Intracellular Ca^{2+} concentration can be effectively elevated by raising the external K^+ concentration (33). Therefore we exposed cultured chromaffin cells to increasing concentrations

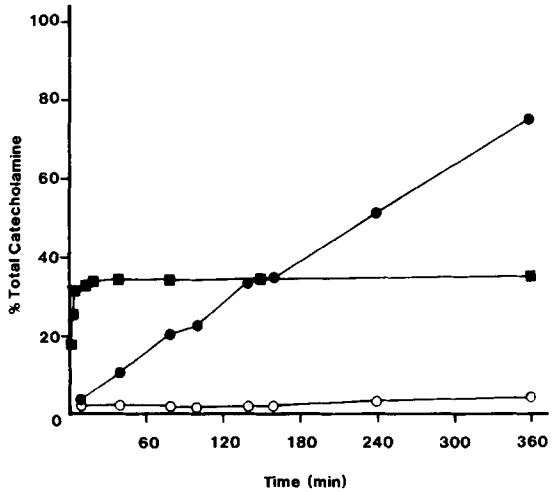


FIGURE 7 A comparison of the time course of catecholamine release from cultured chromaffin cells stimulated by monensin (10^{-6} M, ●) and carbachol (10^{-4} M, ■). Basal levels of catecholamine release are represented by open circles (○). Values are mean ± SEM for three culture wells; error bars are omitted as the symbol size exceeds the length of the bar.

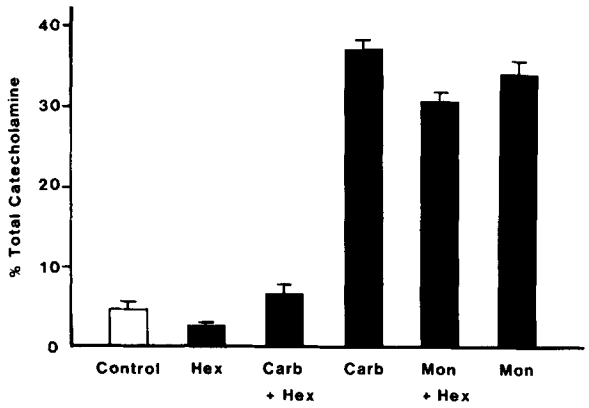


FIGURE 8 The effect of blocking the acetylcholine receptor by pretreating chromaffin cell cultures with hexamethonium (10^{-3} M). While the carbachol (10^{-4} M for 20 min) response was reduced to control levels in the presence of hexamethonium, the response to monensin (10^{-6} M for 150 min) was not substantially altered by pretreatment with hexamethonium. Values are mean ± SEM for three culture wells.

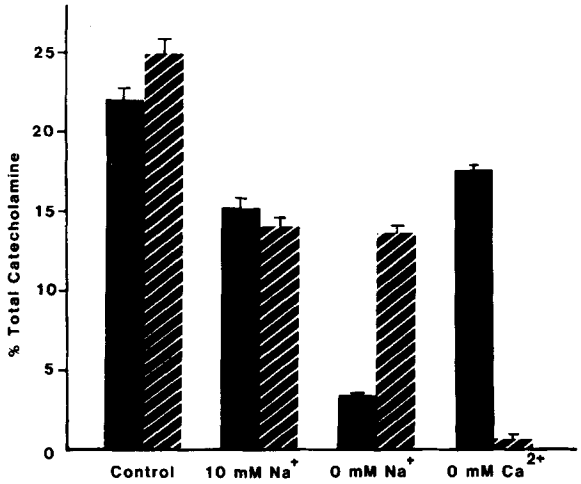


FIGURE 9 The effect of Na^+ and Ca^{2+} concentration on catecholamine release from monensin or carbachol stimulated chromaffin cells. Monensin treated cells (10^{-6} M for 150 min) are represented by a solid bar (■), carbachol treated cells (10^{-4} M for 20 min) are represented by a diagonally striped bar (▨). Control levels of Na^+ and Ca^{2+} were 156 mM and 2.2 mM respectively. In the 10 mM and 0 mM Na^+ solutions, Na^+ was replaced with an osmotic equivalent of sucrose. Values are mean ± SEM for three culture wells.

of K^+ for 2.5 h (Fig. 10). Increasing the K^+ concentration from 5.6 mM (standard Locke's) to 35 mM stimulates a 10–15% increase in catecholamine secretion. No additional release is stimulated by higher concentrations of K^+. This effect can be ascribed to the depolarizing effect of raising extracellular K^+. The K^+-stimulated release is independent of time and does not significantly exceed the amount observed after the standard 20-min exposure.

After a 45-min lag, ouabain (10^{-6} M), which increases intracellular Na^+ and thereby mimics monensin, triggers the release of 86% of chromaffin cell catecholamine in ~4–5 h (Fig. 11). This effect is essentially equivalent to the effect of monensin in the same time period. Ouabain in the presence of monensin produces an accentuated response that parallels the linear monensin-stimulated secretion and results in ~94% catecholamine release after 5 h. This data supports the hypothesis that raising intracellular Na^+ stimulates the release of catecholamines.

The cytoplasm of monensin-treated chromaffin cells is dominated by numerous, smooth-surface vacuoles of various sizes. The vacuoles are noticeably larger than the chromaffin granules and often contain flocculent material. After a 4-h exposure to monensin, several washes with Locke's medium, and return to normal culture conditions in DME for 48 h, these cytoplasmic vacuoles were no longer a prominent cytoplasmic feature. However, these cells did not regain a normal chromaffin granule population and did not release catecholamines regardless of ionic, cholinergic, or ionophore-mediated stimulation.

Comparison with Secretion Mediated by Calcium Ionophores

Since secretion is presumed to be ultimately a Ca^{2+}-mediated response, we compared the monensin response to one obtained by raising the intracellular Ca^{2+} concentration with the calcium-ionophores, ionomycin and A23187. Concentration response curves of A23187, ionomycin, and monensin are shown in Fig. 12. Catecholamine release was determined after 2.5 h exposure to the ionophores. Monensin is effective within a range of 10^{-8} to 3×10^{-6} M, with the maximal effect obtained at 10^{-6} M. Higher concentrations of A23187 and ionomycin

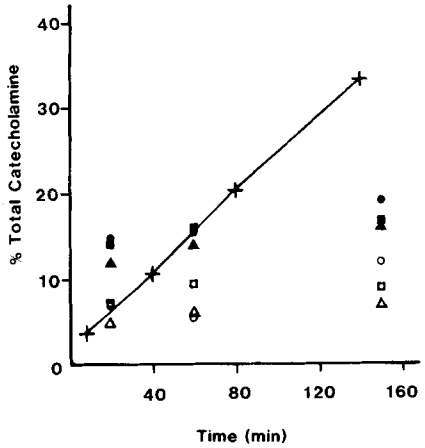


FIGURE 10 A comparison of the time course of catecholamine release from cultured chromaffin cells stimulated by monensin (10^{-6} M, +) and various concentrations of K^+ (5.6–110 mM). 5.6 mM K^+ (△, control); 10 mM K^+ (○); 20 mM K^+ (□); 35 mM K^+ (▲); 56 mM K^+ (●); 110 mM K^+ (■). Release from the cells treated with 35 to 110 mM K^+ was substantially greater than from 5.6 to 20 mM K^+ at all time points. Values are mean ± SEM for three culture wells; error bars are omitted as the symbol size exceeds the length of the bar.

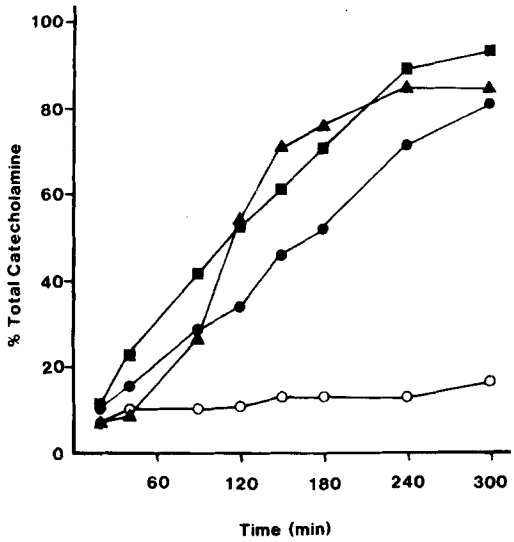


FIGURE 11 A comparison of ouabain- and monensin-stimulated catecholamine release from cultured chromaffin cells. The time course of monensin (10^{-6} M, ●) stimulated catecholamine release is compared to ouabain (10^{-6} M, ▲) and monensin plus ouabain (both drug concentrations were 10^{-6} M, ■). Basal levels of catecholamine secretion are represented by open circles (○). Values are mean ± SEM for three culture wells; error bars are omitted as the symbol size exceeds the length of the bar.

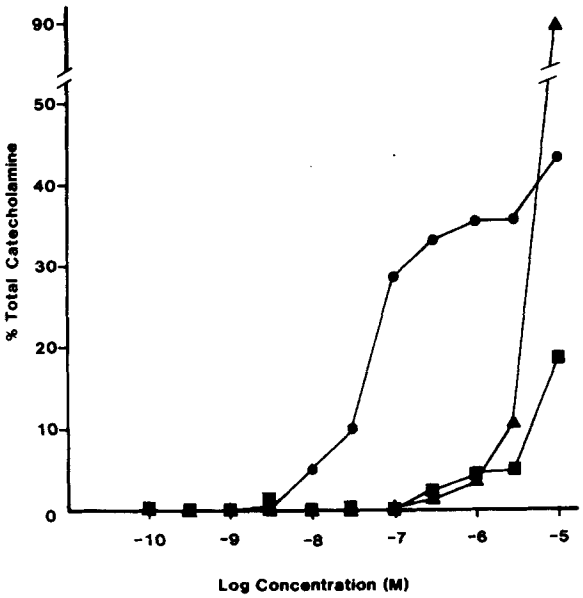


FIGURE 12 A log dose response curve comparing the release of catecholamines from cultured chromaffin cells stimulated by monensin (●), ionomycin (▲), and A23187 (■). The exposure time for all treatments was 150 min. Each experimental value was normalized by subtraction of the corresponding control value. Values are mean ± SEM for three culture wells; error bars are omitted as the symbol size exceeds the length of the bar.

are necessary to stimulate catecholamine secretion, with the effective range for ionomycin from 3×10^{-7} to 3×10^{-6} M and for A23187 from 3×10^{-7} to 10^{-5} M. We do not detect LDH release from cultured chromaffin cells exposed to concentrations of monensin below 3×10^{-6} M, but at concentrations of 10^{-5} M and above, LDH was released in parallel with catecholamines. This implies that the second phase of catecholamine release in the presence of 10^{-5} M monensin is the result of cell damage. The ionophores are amphipathic molecules

(40), and as such would be predicted to possess detergent properties. Thus, at the higher ionophore concentrations, particularly those used in the case of the divalent ionophores, it is difficult to distinguish between a true ionophore-mediated cation transport triggering secretion and release of a secretory product by damage to the cellular and intracellular membranes.

The kinetics of monensin-stimulated catecholamine release were compared to the kinetics of ionomycin and A23187-stimulated release (Fig. 13). Monensin (10^{-6} M) causes a linear release of catecholamines over the 4-h incubation period, with an ultimate release of ~80% of the total catecholamines. Secretion in the presence of ionomycin (3×10^{-6} M) is also quasi-linear over the 4-h period, but secretion does not exceed 24%. A23187 (10^{-5} M) response reaches a maximum at 2 h with about a 22% release of catecholamines.

DISCUSSION

According to the model of stimulus-secretion coupling (41), acetylcholine (the stimulus) binds to cell surface acetylcholine receptors and membrane permeability is thereby altered so that Na^+ and Ca^{2+} ions enter the cell and K^+ ions exit. The resulting increase in intracellular Ca^{2+} activity then triggers exocytosis, wherein secretory granule membranes fuse with the plasma membrane and discharge granule contents (catecholamines) into the extracellular medium (42). Williams (15) has suggested that the Na^+ influx, stimulated by receptor activation, initiates the release of stored Ca^{2+} into the cytoplasm, which in turn, triggers the exocytotic release of the secretory product. Pressman and de Guzman (23) suggested that an ionophore-mediated rise in intracellular Na^+ would promote a similar effect.

To effectively evaluate the role of Na^+ in the secretory process, we used monensin to by-pass receptor activation and directly elevate the intracellular Na^+ concentration of cultured chromaffin cells. By imitating the response to a physiological stimulus, carbachol-stimulated catecholamine release provides a baseline against which to evaluate monensin-induced secretion. Carbachol stimulates a rapid, transient (10–20 min after exposure) release of 25–35% of total catecholamine. In contrast, monensin stimulates a slow linear release which attains comparable catecholamine secretion (25–35%) within 2.5 h and 75–90% release in 6 h. This pattern of linear catecholamine secretion is obtained whether the cell is exposed to monensin for only 20 min or continuously throughout a 5- to 6-h period.

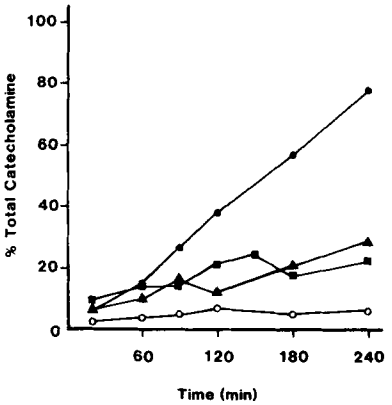


FIGURE 13 The time course of catecholamine release from cultured chromaffin cells stimulated by monensin (10^{-6} M, ●), ionomycin (3×10^{-6} M, ▲), and A23187 (10^{-5} M, ■). The basal level of catecholamine secretion is indicated by open circles (○). Values are mean ± SEM for three culture wells; error bars are omitted as the symbol size exceeds the length of the bar.

The inability of hexamethonium to block monensin-stimulated secretion demonstrates that the action of monensin, unlike carbachol, is independent of cholinergic receptors.

A clear distinction between carbachol and monensin-stimulated catecholamine release is also demonstrated by their requirements for specific extracellular ions. Our results demonstrate that the response to carbachol is dependent on extracellular Ca^{2+} and partially dependent on extracellular Na^+. This result is expected since cholinergic stimulation evokes secretion through mechanisms which cause Ca^{2+}, and to a lesser extent Na^+ (27), to be transported into the cell. In contrast, monensin-stimulated secretion is relatively independent of extracellular Ca^{2+} and dependent on extracellular Na^+. Monensin-stimulated secretion in other cell systems has also been reported to be Na^+-dependent and Ca^{2+}-independent (7, 43, 44). Watson et al. (44) reported that monensin-stimulated amylase release was associated with enhanced $^{22}Na^+$ uptake and $^{45}Ca^{2+}$ efflux, but in the absence of extracellular Na^+, secretion was diminished and $^{45}Ca^{2+}$ efflux not observed. This evidence suggests that Na^+ is somehow linked to the mobilization of bound intracellular Ca^{2+}.

When the Na^+ pump of the chromaffin cell plasma membrane is blocked by ouabain, the observed secretory response is very similar to that produced by monensin. The same response was reported even in the absence of extracellular Ca^{2+} (20). Both veratridine, which increases the permeability of cell membranes to Na^+, and ouabain stimulate insulin release from pancreatic β-cells (18). Veratridine and ouabain also trigger secretion even in the absence of extracellular Ca^{2+} (14, 18). Incubation of sliced adrenal glands in the presence of Na^+-loaded liposomes resulted in a significant increase in catecholamine secretion, suggesting that intracellular Na^+-induced catecholamine release through the mobilization of intracellular Ca^{2+} stores (12). It is interesting to note that Na^+-loaded liposomes are virtually as effective as Ca^{2+}-loaded liposomes in stimulating catecholamine release. Lowe et al. (14) suggest that Na^+, by releasing Ca^{2+} from intracellular pools, acts as a "second messenger" in exocytotic release processes. It is our opinion, however, that the artificial elevation of intracellular Na^+ with monensin or ouabain greatly exceeds the level of Na^+ that would be delivered by a secretagogue-stimulated action potential. Thus, it is unlikely that the mechanism of receptor-mediated secretion in chromaffin cells is solely dependent on Na^+ influx. This proposition is supported by the fact that if chromaffin cells are exposed to carbachol, in the absence of extracellular Ca^{2+}, there is no consequent exocytosis (25).

Assuming that increased intracellular Na^+ acts by mobilization of intracellular Ca^{2+}, one might predict that direct elevation of the intracellular Ca^{2+} concentration with Ca^{2+} ionophores would have an equivalent effect on secretion. However, ionomycin and A23187 are much less effective in stimulating catecholamine release than monensin or ouabain. Monensin stimulates an 80% release of catecholamine, whereas ionomycin and A23187 only stimulate a 20–24% release. Monensin also stimulates catecholamine secretion at much lower concentrations than do the calcium ionophores, exerting its effect over a range of 10^{-8} to 3×10^{-6} M, with a maximum at 10^{-6} M. Apparently, the Na^+-stimulated rise in intracellular Ca^{2+} activity is considerably more effective in stimulating secretion than is the direct elevation of intracellular Ca^{2+} by an ionophore-mediated influx of extracellular Ca^{2+}. The carbachol response is also greater than that estimated by the calcium ionophores (25–35% vs. 20–24% release). This implies that the receptor-mediated response also involves elements

other than a simple rise in intracellular Ca^{2+} concentration.

A second phase of monensin-stimulated catecholamine release, initiated by concentrations of 10^{-5} M and greater, is paralleled by the release of LDH. We interpret the second phase of this biphasic response to reflect leakage of catecholamines, as a result of cell membrane damage due to detergency effects, rather than true catecholamine secretion. Although Kilpatrick et al. (5) report that ionomycin (10^{-5} M) stimulates a linear release of catecholamines from cultured chromaffin cells, we found the effect of ionomycin to be biphasic. Below 3×10^{-6} M it caused a slow linear catecholamine release; above this concentration catecholamine release was sudden and extensive which we interpret as a toxic effect. In pheochromocytoma cells, ionomycin causes catecholamine secretion over a very limited concentration range (10^{-7} to 2×10^{-6} M), above which catecholamine release is accompanied by the release of LDH from the cells (7). In pancreatic acinar cells, A23187 stimulates secretion over a similarly restricted concentration range above which amylase release is paralleled by LDH release (10). In light of these findings, we suggest that great caution be taken in interpreting results obtained with high concentrations of ionophores.

The vacuolated appearance of cells exposed to monensin for 2.5 h is similar to the morphology of other secretory cells exposed to the ionophores, lasalocid, monensin, and A23187 (45–49). Although we do not have evidence to demonstrate the origin of these vacuoles, it has been suggested in other secretory cells exposed to monensin, that such vacuoles originate in the Golgi complex. These vacuoles are thought to arise as a result of ongoing vesicular traffic during secretory granule formation (45, 46, 49). Even though monensin-treated cells regain their normal morphological appearance once they are returned to standard culture conditions, the chromaffin granule population remains reduced. This may suggest that exposure to monensin somehow alters secretory granule formation in these cells as well.

High K^+, carbachol, and Ba^{2+} stimulate the release of 15, 30, and 50% of the total catecholamine content, respectively. The maximum catecholamine secretion obtained with three successive carbachol applications (47%) is similar to that released in the presence of Ba^{2+} (50%). Additional Ba^{2+} or carbachol stimulations did not elicit additional catecholamine secretion. Likewise, Poisner et al. (50) have shown that repeated acetylcholine stimulation of intact adrenal glands will not trigger additional catecholamine secretion above a certain level (41%). These results suggest that only 40–50% of the granules in a cell are both competent and available for exocytosis. Since exocytosis can occur over the entire surface of the cells, we expected to observe an absence of granules adjacent to the plasma membrane. Such an observation would support the hypothesis that the rate-limiting event in secretion is the movement of secretory granules to the cell surface. However, the homogeneous distribution of granules throughout the cytoplasm of Ba^{2+}- and carbachol-stimulated cells does not support this hypothesis. Perhaps the availability of competent granules and exocytotic sites on the plasma membrane limit secretion.

The amount of secretion stimulated by monensin is markedly greater than that elicited by either cholinergic or direct ionic stimulation. Monensin-induced catecholamine release exceeds 50% at 4 h. The ability of monensin to elicit secretion that eventually exceeds the ~50% release stimulated by Ba^{2+} and multiple carbachol applications may be a reflection of the ability of the ionophore to affect the mobilization of granules to the plasma membrane as well as stimulate exocytosis. Since monensin is presumed to indirectly elevate intracellular Ca^{2+} activity (51), we propose that monensin may modulate cytoskeletal-secretory granule interactions that occur during intracellular transport, thereby stimulating the movement of exocytotically competent granules to the plasma membrane. The continuous release of catecholamines in the presence of monensin requires the continual translocation of secretory granules to the plasma membrane. This is very different from the immediate and rapid response to physiologically active agents such as acetylcholine.

We conclude that the role of Na^+ in catecholamine secretion is to facilitate the stimulatory effect of increased intracellular Ca^{2+} on exocytosis. The remarkable level of secretion promoted by monensin-augmented intracellular Na^+ may be indicative of an action of intracellular Na^+ of physiological significance. Conceivably, changes in cytosolic Na^+ could alter the rise in Ca^{2+} activity produced by a secretagogue-mediated rise in cytosolic Ca^{2+} concentration. On the whole organism level, cell-type specific fluctuations in intracellular Na^+ levels, under metabolic and/or hormonal control, might act to attenuate or enhance the response of cells to a secretagogue and thus account for the differential response of various cell types to the same secretagogue.

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