The plasma membrane of cultured chromaffin cells from bovine adrenal medulla was rendered leaky by incubation in low concentrations of digitonin. Digitonin (20 μM) induced Ca²⁺-dependent release of 10–20% of the catecholamine in the presence of 10 μM Ca²⁺ without addition of secretagogue. Half-maximal catecholamine release occurred at approximately 1 μM Ca²⁺. Mg²⁺ could not substitute for Ca²⁺. Cells incubated with digitonin rapidly lost their ability to exclude trypan blue. Digitonin caused release of the cytosolic markers lactic dehydrogenase and phenylethanolamine-N-methyltransferase, but, in contrast to release of catecholamine, the release of the cytosolic proteins was inhibited by Ca²⁺. Soluble dopamine-β-hydroxylase, a protein marker of granule contents, was released proportionally with catecholamine in a Ca²⁺ dependent manner. Catecholamine release was optimal in solutions containing MgATP. Hence, digitonin-treated cells, although they lose soluble cytosolic proteins and presumably low molecular weight cytosolic constituents, maintain the Ca²⁺-dependent reactions of exocytosis in the presence of MgATP. Digitonin-treated chromaffin cells may be a powerful system in which to study the biochemical mechanisms underlying exocytosis.

The secretion of neurotransmitters and many prepackaged hormones is triggered by the influx of Ca²⁺ which by unknown mechanisms causes storage vesicles to fuse with the plasma membrane and release their soluble contents to the cell exterior (exocytosis). This sequence of events was first determined biochemically in studies concerning the mechanism of catecholamine secretion from the adrenal medulla. The work of Douglas, Rubin, Kirshner, Viveros, and others (see Ref. 1 for review) demonstrated that upon cholinergic stimulation, Ca²⁺ permeated the plasma membrane of freshly suspended adrenal medullary cells by exposing cell suspensions to high voltage discharges (e.g., 100–120 kV/cm). This caused localized dielectric breakdown of the plasma membrane. They found that micromolar Ca²⁺ without secretagogue induced exocytosis and that if cells were allowed to incubate for longer than 5 min after the high voltage treatment, there was an MgATP dependency for secretion. The permeability change was somewhat selective since although ATP became permeable, the cytosolic protein lactic dehydrogenase only slowly exited from the cells.

Digitonin, a steroid glycoside which interacts specifically with squalene, increases the membrane permeability of cells to Ca²⁺ and Mg²⁺ (5–9). In the present study, we have used digitonin to increase the permeability of the plasma membrane of cultured chromaffin cells from the adrenal medulla. We demonstrate that digitonin-treated cells release catecholamine upon addition of micromolar Ca²⁺ to the medium in the absence of secretagogue in a manner consistent with exocytosis.

Toward the end of this study, we learned of similar ongoing work by Wilson and Kirshner (10), the results of which they have shared with us. Where our experiments overlap, we are in excellent agreement, both in the actual results and in the interpretation of the data. Our studies are being published together (10).

MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in minimal essential medium containing 10% heat-inactivated fetal calf serum as previously described (11, 12). The culture medium contained 100 units/ml of penicillin, 100 μg/ml of streptomycin, 50 μg/ml of gentamycin, and 1.3 μg/ml of Fungizone (Squibb) to prevent bacterial and fungal contamination. The culture medium also contained 10 μM cytochrome arabinoside to inhibit fibroblast growth. Cultures were incubated in bovine arabinoside-free medium for 1 day before each experiment. Cells were usually cultured as monolayers in plastic culture wells (16-mm diameter) at a density of 250,000 cells/cm² and were used in experiments 4–14 days after preparation. There were approximately 40 pmol of catecholamine/million cells. Immediately before an experiment, cells were incubated for 1 h with physiological salt solution containing 145 mM NaCl, 5.5 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES¹ (pH 7.4), and 0.5 mM sodium ascorbate. An experiment was initiated by replacing the medium with new solution. All experiments were performed at 37°C.

The solution used for most of the experiments with digitonin was similar to that of Baker and Knight (3) and contained 139 mM potassium glutamate, 20 mM PIPES (pH 6.5), 5 mM MgCl₂, 5 mM glucose, 0.5 mM ascorbic acid, and either 5 mM EGTA (without Ca²⁺) or 5 mM EGTA and various amounts of calcium. Calcium concentrations in calcium-EGTA buffers were calculated according to Portzehl (13). Mg²⁺ and Ca²⁺ binding to ATP were calculated based upon the data from Nanninga and Kempen (14). In the above solution containing 10 μM free Ca²⁺ (4.43 mM CaCl₂), 4 mM MgCl₂ was bound to ATP.

¹The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

Catecholamine Secretion from Digitonin-treated Adrenal Medullary Chromaffin Cells*

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and the free Mg²⁺ concentration was 1 mM. The calcium-EGTA equilibrium was not significantly altered by the concentrations of Mg²⁺ and ATP that were present.

In most experiments, secretion was monitored by determining the percentage of [³H]norepinephrine released from cells which had been previously incubated in [³H]norepinephrine-containing solution according to Kilpatrick et al. (15). Release of endogenous catecholamine (epinephrine + norepinephrine) was measured according to Holz et al. (12) using the fluorescent method of von Euler and Flooding (16) with internal standards of epinephrine. Dopamine-β-hydroxylase was measured by the method of Nagatsu and Udenfriend (17) in the presence of 3 μM CuSO₄ which gave maximal activity. Soluble dopamine-β-hydroxylase was liberated from cells and chromaffin granules by homogenization in 10 mM Na-HEPES (pH 7.0), 5 mg/ml of bovine serum albumin, and 0.1 mg/ml of catalase and separated from particulate dopamine-β-hydroxylase by centrifugation (35,000 × g × 30 min). Soluble dopamine-β-hydroxylase served as a marker for soluble protein within the storage vesicle. Phenylethanolamine-N-methyltransferase, a specific chromaffin cell cytosolic marker, was measured according to Molinoff et al. (18) using purified S-adenosylmethionine. Both dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase activities were measured in samples from which EDTA and catecholamine had been removed by chromatography with Sephadex G-25 (medium mesh) using columns (0.5 × 10.5 cm) which had been equilibrated with 50 mM NaCl, 10 mM Na-HEPES (pH 7.0). Lactic dehydrogenase was measured according to Kornberg (19). The ability of cells to exclude trypan blue for at least 4 min in solution containing 2 mg/ml of trypan blue was assessed as a measure of plasma membrane integrity. Cells were visualized at ×125 and ×200 magnification.

All reagents were readily available commercially. L-[³H]Norepinephrine (214 Ci/mmole) was obtained from New England Nuclear. S-Adenosyl-L-[¹⁰⁶M]methionine (15 Ci/mmole) was obtained from Amersham Corp. Data were expressed as mean ± standard error of the mean. When the difference between two groups was determined, the standard error of the mean was calculated as (S.E.₁ + S.E.₂)/² where S.E.₁ and S.E.₂ are the standard errors of the mean for each group. Differences between means of groups were tested for significance with the Student's t test.

**RESULTS**

*Effects of Various Digitonin Concentrations on Ca²⁺-dependent Secretion—Digitonin caused a dose-dependent release of [³H]norepinephrine which corresponded to approximately 75% of the total [³H]norepinephrine at 300 μM (Fig. 1A). A Ca²⁺-dependent component of [³H]norepinephrine release occurred between 10 and 100 μM digitonin (Fig. 1B). Catecholamine release was not induced by 10 μM Ca²⁺ in the absence of digitonin.*

In most experiments, secretion was monitored by determining the amount of [³H]norepinephrine released from cells in which the catecholamine stores had been previously labeled with [³H]norepinephrine. [³H]Norepinephrine secretion from intact cells accurately reflects endogenous catecholamine secretion (15). Similarly, [³H]norepinephrine release from cells treated with digitonin closely approximated endogenous catecholamine release (Table I).

**Time Course of Secretion and Effect of Preincubation with Digitonin**—[³H]Norepinephrine release increased with time both in the presence and absence of Ca²⁺ (Fig. 2A). Ca²⁺-dependent secretion began after 1 min and continued for approximately 15 min (Fig. 2A). The 1-min lag time may reflect the time necessary for digitonin to increase significantly the permeability of the plasma membrane. Consistent with this interpretation are the results shown in Fig. 3. Catecholamine cells preincubated with digitonin (20 μM) in the absence of Ca²⁺ for various times were subsequently incubated without digitonin in the presence or absence of Ca²⁺ (10 μM) for 15 min. A 5-min exposure to digitonin was necessary to obtain maximal Ca²⁺-dependent secretion in the subsequent 15-min test period. In the experiment in Fig. 3, Ca²⁺-dependent secretion from cells preincubated for 5 min with digitonin was approximately equal to Ca²⁺-stimulated secretion from cells during a continuous incubation with digitonin (see the legend to Fig. 3). In three experiments with a 5-min preincubation with digitonin (20 μM) followed by a 15-min incubation without digitonin, Ca²⁺-dependent [³H]norepinephrine release averaged 0.8 that obtained with continuous incubation with digitonin. Thus, it takes several minutes for digitonin to act, but once the plasma membrane becomes leaky, the continued presence of digitonin is unnecessary for maintenance of the increased Ca²⁺ permeability.

**Table I**

*Release of endogenous catecholamine and [³H]norepinephrine from cells*

<table>
<thead>
<tr>
<th>Per cent of total released</th>
<th>Endogenous catecholamine</th>
<th>[³H]Norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Ca²⁺</td>
<td>15.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>10 μM Ca²⁺</td>
<td>31.2 ± 1.6a</td>
</tr>
<tr>
<td>Ca²⁺-dependent release</td>
<td>15.3 ± 1.8</td>
<td>20.5 ± 1.9</td>
</tr>
</tbody>
</table>

a *p < 0.001 versus 0 Ca²⁺.

**Fig. 1. Effect of various concentrations of digitonin on norepinephrine release from chromaffin cells.**

Cells containing [³H]norepinephrine (NE) were incubated in potassium glutamate solution containing 5 mM MgATP and various concentrations of digitonin in the presence or absence of 10 μM Ca²⁺. After 15 min, the solution was removed, and [³H]norepinephrine released into the solution and that remaining in the cells was measured. A, [³H]norepinephrine released into the medium in the presence and absence of 10 μM Ca²⁺. B, difference between release of [³H]norepinephrine in the presence and absence of Ca²⁺. There were three wells/group.
Ca\textsuperscript{2+} Dependency of Secretion—Catecholamine secretion in the presence of 20 \mu M digitonin was responsive to calcium from below 1 to 10 \mu M (Fig. 4). The Ca\textsuperscript{2+} response was specific since the 1 mM free Mg\textsuperscript{2+} that was present in the solutions did not support comparable \textsuperscript{3}Hnorepinephrine release. The Ca\textsuperscript{2+} concentrations are probably within the physiological range for the cytosol and are similar to those which induce secretion from freshly suspended bovine chromaffin cells that had been rendered leaky by high voltage discharges (3). Carbachol-induced secretion in physiological salt solution required approximatively a 500-fold greater Ca\textsuperscript{2+} concentration than Ca\textsuperscript{2+}-dependent secretion from digitonin-permeabilized cells (Fig. 4).

Trypan Blue Staining of Cells—The ability of cells to exclude trypan blue was assessed as one measure of plasma membrane integrity. Cells were incubated for various times in potassium glutamate solution containing 5 mM EDTA with or without 20 \mu M digitonin. After various times, the solution was replaced with potassium glutamate solution containing 5 mM EGTA and 2 mg/ml of trypan blue without digitonin. Fewer than 5% of the cells not incubated in digitonin took up trypan blue. Cells incubated in digitonin progressively lost their ability to exclude trypan blue. After a 1-min incubation in digitonin, approximately 50% of the cells could still exclude trypan blue; cells in clumps were particularly resistant to trypan blue staining. After 2 min, most cells were stained although some cells in clumps did not stain. After incubation for 5 min in digitonin, virtually 100% of the cells lost the ability to exclude trypan blue. These results are qualitatively consistent with

![Image](https://example.com/image.png)
the time course of Ca\textsuperscript{2+}-dependent secretion (Fig. 2) and lactic dehydrogenase release (see below), which both require several minutes incubation with digitonin.

Cells incubated for 5 min with 20 \(\mu\)M digitonin and then incubated for 15 min without digitonin before introduction of trypan blue did not regain their ability to exclude trypan blue. Thus, cells do not rapidly reseal after digitonin treatment, which is also indicated by their continued ability to secrete catecholamine in response to 10 \(\mu\)M Ca\textsuperscript{2+} (Fig. 3).

**Release of Enzyme Markers**—The release of enzymatic markers was measured to investigate further the effects of digitonin on plasma membrane permeability and to investigate the mechanism of Ca\textsuperscript{2+}-dependent secretion from digitonin-treated cells. Lactic dehydrogenase, a cytoplasmic marker of 134,000 daltons, was gradually released from the cells by 20 \(\mu\)M digitonin (Fig. 5A); by 15 min, approximately 50% of the lactic dehydrogenase was released from the cells. Ca\textsuperscript{2+} (10 \(\mu\)M) reduced lactic dehydrogenase release. \([\text{H}]\)Norepinephrine release from the same cells had a different pattern of release. In the absence of Ca\textsuperscript{2+}, 10% of the \([H]\)norepinephrine was released by 2 min and there was little further release by 15 min. In the presence of Ca\textsuperscript{2+} (16 \(\mu\)M), there was a large time-dependent release of \([\text{H}]\)norepinephrine.

The release of \([\text{H}]\)norepinephrine, soluble dopamine-\(\beta\)-hydroxylase (a 290,000-dalton marker for the soluble proteins within chromaffin granules), phenylethanolamine-N-methyltransferase (a 38,000-dalton cytosolic marker specific for chromaffin), and lactic dehydrogenase (a 134,000-dalton cytosolic marker for all cells) was also compared (Table I). In the absence of digitonin, there was virtually no Ca\textsuperscript{2+}-dependent release of \([\text{H}]\)norepinephrine or dopamine-\(\beta\)-hydroxylase (see also Fig. 1A). Although the high potassium concentration probably depolarized the cells, the Ca\textsuperscript{2+} concentration (10 \(\mu\)M) was too low to support significant secretion in the intact cells (12). Virtually no phenylethanolamine-N-methyltransferase or lactic dehydrogenase was released in the absence of digitonin. In the presence of digitonin, little \([\text{H}]\)norepinephrine or soluble dopamine-\(\beta\)-hydroxylase was released in the absence of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} stimulated the release of virtually identical proportions (21 and 23%, respectively) of both cytosolic markers with a small but significant (\(p<0.05\)) decrease in lactic dehydrogenase release in the presence of Ca\textsuperscript{2+}. Although in this experiment Ca\textsuperscript{2+} did not inhibit the release of phenylethanolamine-N-methyltransferase, in another experiment, Ca\textsuperscript{2+} inhibited its release by 20%. Thus, a soluble protein constituent as well as the catecholamine content of the chromaffin granule were released proportionally and specifically in a Ca\textsuperscript{2+}-dependent manner. Digitonin, therefore, did not simply cause Ca\textsuperscript{2+}-dependent lysis of the cells. The data are consistent with digitonin increasing the permeability of the plasma membrane to Ca\textsuperscript{2+} and large molecules without significantly lysing the chromaffin granules or releasing intact granules. Micromolar Ca\textsuperscript{2+} in the medium could then equilibrate across the plasma membrane and thereby initiate exocytosis.

**MgATP Dependency of Secretion**—MgATP was present in all solutions used in the digitonin experiments above. Baker and Knight (3, 4) demonstrated an absolute requirement of secretory ATP for MgATP. In our experiments, optimal secretion required the presence of MgATP. For example, in cells preincubated in 20 \(\mu\)M digitonin without Ca\textsuperscript{2+} for 5 min in the presence or absence of 5 mM MgATP and then incubated for 15 min without digitonin in the continued presence or absence of 5 mM MgATP with or without Ca\textsuperscript{2+} (similar protocol to that of Fig. 3), Ca\textsuperscript{2+}-dependent release during the 15-min incubation was 24.4 \(\pm\) 0.8% (\(n=3\)) in the presence of 5 mM MgATP and 4.2 \(\pm\) 1.1% (\(n=3\)) in the absence of MgATP. However, the extent of the MgATP dependency of secretion has been variable. In some experiments, omission of ATP or Mg\textsuperscript{2+} and ATP resulted in only a 50% inhibition of secretion. It is possible that residual ATP from the cells was responsible for the variability. The nucleotide dependency is currently being more fully investigated.
DISCUSSION

Digitonin increases membrane permeability by interacting with cholesterol in membranes. Because both the chromaffin granule membrane and the plasma membrane contain cholesterol (20), one expects digitonin to increase the permeability of both membranes. A crucial aspect of the action of digitonin on cultured chromaffin cells is that there exists a concentration of digitonin and an incubation time with digitonin which preferentially render the plasma membrane leaky. Incubation of chromaffin cells with 20 \( \mu \)M digitonin for 2-15 min induced substantial leakage from the cytosol of phenylethanolamine-N-methyltransferase (38,000 daltons) and lactic dehydrogenase (134,000 daltons). Cells retained as much as 97% of catecholamine (170 daltons) and soluble dopamine-\( \beta \)-hydroxylase (290,000 daltons), which are substituents of the chromaffin granule. The data also indicate that intact chromaffin granules (2,000-A diameter) do not cross the leaky plasma membrane.

Introduction of micromolar Ca\( ^{2+} \) in the medium released from cells approximately 20% of the total catecholamine and soluble dopamine-\( \beta \)-hydroxylase, without increasing the release of the cytosolic markers phenylethanolamine-N-methyltransferase and lactic dehydrogenase. In most experiments, the release of the cytosolic markers was decreased somewhat by 10 \( \mu \)M Ca\( ^{2+} \). The presence of MgATP was required for optimal catecholamine secretion. The simplest interpretation of the data is that micromolar Ca\( ^{2+} \) in the medium equilibrated across the plasma membrane of digitonin-treated cells and initiated exocytosis. However, because digitonin treatment allowed large proteins to exit from the cells, it is also possible that Ca\( ^{2+} \) induced intracellular lysis of the granules with subsequent leakage of both \(^3\text{H}\)norepinephrine and soluble dopamine-\( \beta \)-hydroxylase into the medium. This mechanism would require that the 290,000-dalton dopamine-\( \beta \)-hydroxylase permeate the leaky plasma membrane at the same rate as the 170-dalton \(^3\text{H}\)norepinephrine. Although this is unlikely, definitive proof that in digitonin-treated cells the mechanism of release induced by micromolar Ca\( ^{2+} \) is exocytosis may require the demonstration that the granule membrane is incorporated into the plasma membrane.

The experiments with digitonin-treated cells demonstrate the feasibility of increasing the plasma membrane permeability to molecules as large as 134,000 daltons and yet at the same time maintaining essential Ca\( ^{2+} \)-dependent reactions of exocytosis or of processes closely related to exocytosis. The technique may allow the direct intracellular introduction of drugs and other agents that may interfere with or alter exocytosis. The substantial increase of plasma membrane permeability to lactic dehydrogenase induced by digitonin contrasts with the permeability change induced by high voltage discharges (3,4) which results in only a small leakage of lactic dehydrogenase. Digitonin treatment of hepatocytes allows IgG (150,000-dalton) entry (7). Thus, digitonin treatment of chromaffin cells may permit for the first time the introduction of antibodies into functioning cells which are directed against suspected components of the exocytotic process, and may prove to be a powerful technique for the elucidation of the biochemical reactions involved in exocytosis.

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