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Relationship Between Ca^{2+} Uptake and Catecholamine Secretion in Primary Dissociated Cultures of Adrenal Medulla

Ronald W. Holz, Ruth A. Senter, and Roy A. Frye

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

Abstract: Carbachol or elevated K^+ stimulated $^{45}Ca^{2+}$ uptake into chromaffin cells two- to fourfold. The uptake was stimulated by cholinergic drugs with nicotinic activity, but not by those with only muscarinic activity. Ca^{2+} uptake and catecholamine secretion induced by the mixed nicotinic-muscarinic agonist carbachol were inhibited by the nicotinic antagonist mecamylamine, but not by the muscarinic antagonist atropine. Significant Ca^{2+} uptake occurred within 15 s of stimulation by carbachol or elevated K^+ at a time before catecholamine secretion was readily detected. At later times the time course of secretion induced by carbachol or elevated K^+ was similar to that of Ca^{2+} uptake. There was a close correlation between Ca^{2+} uptake and catecholamine secretion at various concentrations of Ca^{2+}. The concentration dependencies for inhibition of both processes by Mg^{2+} or Cd^{2+} were similar. Ca^{2+} uptake saturated with increasing Ca^{2+} concentrations, with an apparent K_m for both carbachol-induced and elevated K^+-induced Ca^{2+} uptake of approximately 2 m*M*. The Ca^{2+} dependency, however, was different for the two stimuli. The studies provide strong support for the notion that Ca^{2+} entry and a presumed increase in cytosolic Ca^{2+} concentration respectively initiates and maintains secretion. They also provide evidence for the existence of saturable, intracellular, Ca^{2+}-dependent processes associated with catecholamine secretion. Ca^{2+} entry may, in addition, enhance nicotinic receptor desensitization and may cause inactivation of voltage-sensitive Ca^{2+} channels. **Key Words:** Ca^{2+} uptake—Catecholamines—Adrenal medulla—Carbachol—Nicotinic-muscarinic agonist—Cell culture. **Holz R. W. et al.** Relationship between Ca^{2+} uptake and catecholamine secretion in primary dissociated cultures of adrenal medulla. *J. Neurochem.* **39**, 635–646 (1982).

Secretion of "prepackaged" hormones and neurotransmitters generally occurs by the process of exocytosis. Studies on the perfused adrenal medulla and adrenal medullary slices helped define the process and demonstrated that in this tissue Ca^{2+} influx (and a presumed increase in cytosolic Ca^{2+} concentration) initiated secretion (see Douglas, 1975, and Viveros, 1975, for reviews). Although these studies led to a greatly increased understanding of secretion and served as a model for studies in other secretory systems, they were often limited in their time resolution and their ability to quantitate events occurring simultaneously with secretion. These limitations have been overcome with the development of primary dissociated monolayer cultures of bovine adrenal medullary chromaffin cells (Fenwick et al., 1978; Livett et al., 1979; Kilpatrick et al., 1980). In the present study we have investigated the relationship between Ca^{2+} uptake and catecholamine secretion in cultured chromaffin cells. The results demon-

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Address correspondence and reprint requests to Dr. Ronald W. Holz, Department of Pharmacology, University of Michigan Medical School, M6322 Medical Sciences I, Ann Arbor, MI 48109, U.S.A.

Abbreviation used: PSS, Physiological salt solution.

strate that secretion is well correlated with Ca^{2+} uptake, and that Ca^{2+} permeability is controlled by a number of different parameters.

MATERIALS AND METHODS

Chromaffin cells, disaggregated from bovine adrenal medulla, were added to 16-mm-diameter uncoated culture wells (approximately 500,000 cells/well) containing 1 ml of modified Eagle's Medium supplemented with 10% heat-inactivated fetal calf serum, 10 μM 5-fluorodeoxyuridine (to inhibit cell division and fibroblast proliferation), 50 μg/ml gentamycin, and 1.3 μg/ml Fungizone (3-6). After 4–8 days at 34°C in 5% CO_2–95% air, chromaffin cells formed monolayers containing 12–20 nmol catecholamine per well. In some preparations, a Percoll (Pharmacia, Piscataway, NJ) gradient was employed for further purification of the chromaffin cells (Kilpatrick et al., 1980), but since there was no apparent effect on experimental results, this step was discontinued. Experiments were performed (unless otherwise indicated) at 25°C in 0.36 or 0.5 ml physiological salt solution containing 142 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 5.6 mM glucose, 0.5 mM sodium ascorbate, and 15 mM HEPES buffer (pH 7.4). In some experiments PSS was modified to contain 56 mM KCl and 92 mM NaCl. To terminate secretion experiments, test solutions were removed and added to concentrated trichloroacetic acid (50%) to yield a 5% concentration of acid. Trichloroacetic acid, 5% (0.5 ml), was added to some wells to liberate catecholamine in the cells. Catecholamines (norepinephrine + epinephrine) released into the medium and catecholamines in the extract of cells were measured by the method of von Euler and Floding (1955). Data were expressed as percentage of the total catecholamine released into the medium.

$^{45}Ca^{2+}$ influx (Ca^{2+} uptake) was measured by incubating the cells in PSS containing $^{45}CaCl_2$ (usually 0.2 μCi/ml). After a given length of time the radioactive medium was aspirated and saved for catecholamine determinations. Unless otherwise indicated, cells were washed three times with 1 ml of nonradioactive PSS at 0–4°C. A solution (0.5 ml) containing 1% Triton X-100 and 1 mM EGTA was then added to each well. The radioactivity liberated from the cells was counted in 4 ml ACS (Amersham, Chicago, Il.) scintillation counting solution. Data were usually expressed as nmoles of Ca^{2+} taken up by the cells.

Ca^{2+} content of the cells was determined by digesting cells for 2 days in concentrated HNO_3. The Ca^{2+} content of the extracts was measured in a Varian AA 375 atomic absorption spectrophotometer.

$^{45}CaCl_2$ (37.4 Ci/g atom) was obtained from New England Nuclear, Boston, MA. Eagle's minimum essential medium and heat-inactivated fetal calf serum were obtained from GIBCO, Grand Island, NY. 5-Fluorodeoxyuridine, atropine, *d*-tubocurarine, bethanechol, muscarine, methacholine, nicotine, and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) were obtained from Sigma Chemical Co., St. Louis, MO. Fungizone was obtained from Squibb, Princeton, NJ. Gentamycin was purchased from Microbiological Associates, Bethesda, MD.

Data are expressed as the mean ± SEM, and significance was tested by Student's *t*-test. When the difference between two groups was determined, SEM was generally calculated as $(SEM_1^2 + SEM_2^2)^{1/2}$, where SEM_1 and SEM_2 are the standard errors of the mean for each group. Experiments presented were repeated at least twice.

RESULTS

$^{45}Ca^{2+}$ uptake procedure

After cells were incubated in $^{45}Ca^{2+}$-containing solution, it was necessary to wash the monolayers with nonradioactive solution to remove $^{45}Ca^{2+}$ in the medium and $^{45}Ca^{2+}$ loosely bound to the exterior of the cells. It was anticipated that the $^{45}Ca^{2+}$ taken up into the cells would be rapidly bound or sequestered and would remain within the cells during rapid washes. As discussed below, depolarization caused by elevated K^+-containing solution (56 mM K^+) induced Ca^{2+} uptake. The effect of the number of washes on the measured depolarization-induced $^{45}Ca^{2+}$ uptake was investigated (Table 1). Each of four washes reduced the $^{45}Ca^{2+}$ associated with the cells. However, the increment of $^{45}Ca^{2+}$ uptake due to depolarization was unaltered (far right column). The main effect of increased number of washes was to reduce the $^{45}Ca^{2+}$ uptake that was unrelated to stimulated secretion. The decrement may represent $^{45}Ca^{2+}$ not within the cells. Because the fourth wash caused a relatively small decrease (28%) in $^{45}Ca^{2+}$ associated with the cells in 5.6 mM K^+, in the following experiments cells were washed three times with nonradioactive Ca^{2+} PSS.

Stimulated $^{45}Ca^{2+}$ uptake was approximately 0.5–1 nmol/well. The water space of the cell monolayer determined by 3-*O*-methyl-D-glucose equilibration (Kletzien et al., 1975) was 0.3–0.5 μl. Hence, if $^{45}Ca^{2+}$ uptake represented net uptake, then the nominal increase in intracellular Ca^{2+} concentration was approximately 2 mM. Because free cytosolic Ca^{2+} concentration is generally less than 10 μM, even in cells in which Ca^{2+} permeability has been increased, it is likely that most of the $^{45}Ca^{2+}$ taken up was, indeed, rapidly sequestered.

TABLE 1. *Effect of the number of washes on the measurement of depolarization-induced Ca^{2+} uptake*

Number of washes	^{45}Ca uptake, cpm		
	5.6 mM K^+	56 mM K^+	Depolarization-induced uptake
1	478 ± 29	615 ± 30	137 ± 42
2	153 ± 9	302 ± 7	148 ± 11
3	65 ± 2	222 ± 9	157 ± 9
4	47 ± 4	187 ± 13	140 ± 14

Cells were incubated for 2 min in 0.5 ml of either 5.6 mM K^+-containing solution (Ca^{2+} PSS) or 56 mM K^+-containing solution (Na^+ concentration was reduced to 92 mM) at 25°C. The solutions also contained 4.06×10^5 cpm $^{45}Ca^{2+}$/ml. At the end of the incubation the cells were washed for the indicated number of times with 1 ml of nonradioactive Ca^{2+} PSS at 4°C. $^{45}Ca^{2+}$ was then extracted and the radioactivity determined as described in Materials and Methods. The depolarization-induced ^{45}Ca uptake (far right column) is the difference between $^{45}Ca^{2+}$ uptake in 56 mM K^+- and 5.6 mM K^+-containing solution.

Relationship between carbachol-induced secretion and Ca^{2+} uptake

The rate of carbachol-induced catecholamine secretion was maximal during the initial 2 min, slowed between 2 min and 5 min, and ceased after 5 min (Fig. 1A). Significant carbachol-stimulated Ca^{2+} uptake could be detected at 15 s before the occurrence of significant catecholamine secretion. The rate of carbachol-induced Ca^{2+} uptake was maximal between 0 and 1 min, and slowed after 5 min. The total carbachol-dependent Ca^{2+} uptake from the medium actually declined between 5 and 15 min. The decline may reflect the efflux of Ca^{2+} previously taken up and not compensated for by Ca^{2+} influx. To estimate more accurately the rate of Ca^{2+} influx at times after 2 min, $^{45}Ca^{2+}$-containing solution was introduced for the periods 5–7 min and 15–17 min after exposure to carbachol. The rate of carbachol-induced Ca^{2+} uptake was maximal 0–2 minutes after exposure to carbachol, but was statistically insignificant from controls at 5–7 min and 15–17 min after exposure to agonist (data not shown). The results are consistent with the notion that secretion from chromaffin cells is stimulated by elevated cytosolic Ca^{2+} concentration, which occurs because of Ca^{2+} entry; they indicate that Ca^{2+} influx may, in fact, precede secretion.

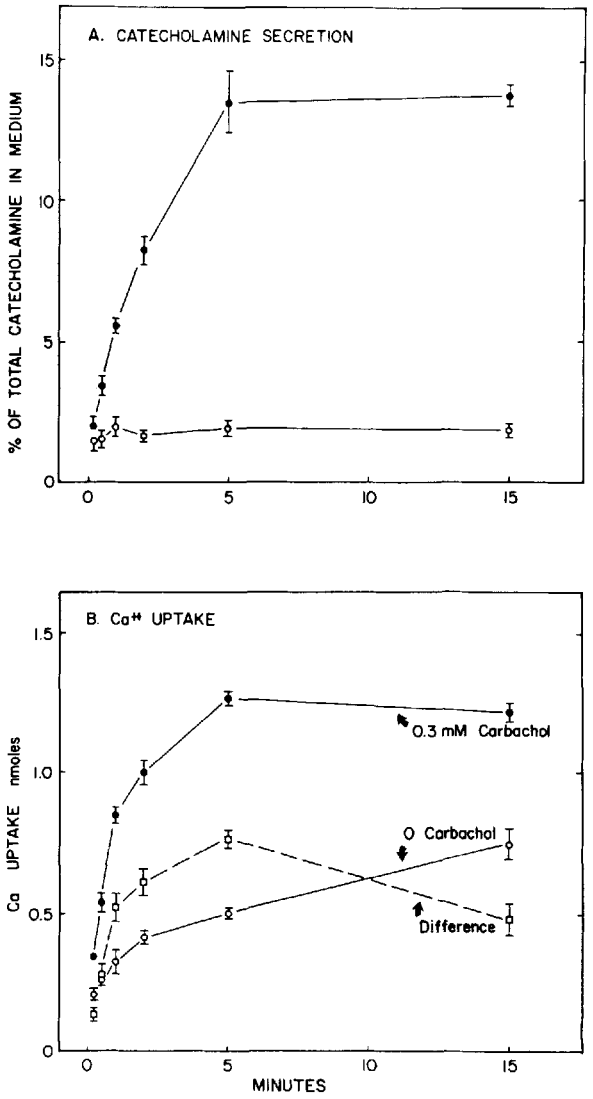

FIG. 1. Time course of carbachol-induced catecholamine secretion **(A)** and Ca^{2+} uptake **(B).** Cells were incubated in solution containing $^{45}Ca^{2+}$ in the presence (●) or absence (○) of 0.3 m*M* carbachol. The solution was aspirated at various times and saved for measurement of catecholamine released from the cells. Catecholamine secretion and Ca^{2+} uptake into cells were determined as described in Materials and Methods. The dashed line in B represents the difference between Ca^{2+} uptake in the presence and absence of carbachol. There were four wells per group. The standard errors of the mean bars of some points were smaller than the point symbols and were omitted.

Carbachol dose-response curve

The effects of various concentrations of carbachol on catecholamine secretion and Ca^{2+} uptake were investigated (Fig. 2). Neither carbachol-dependent secretion nor Ca^{2+} uptake was detected at 10^{-5} *M;* both were significant with 0.1 m*M* carbachol. Secretion was maximal by 0.3 m*M*, whereas Ca^{2+} uptake was maximal with 1 m*M* carbachol. Thus, at low concentrations of carbachol there was a correlation between carbachol-induced Ca^{2+} uptake and catecholamine secretion. However, at concentrations of carbachol greater than 0.3 m*M*, a fraction of the carbachol-induced Ca^{2+} entry had no effect on catecholamine secretion. Secretion measured after 15 min exposure to carbachol was also maximal by 0.3 m*M*.

Pharmacology of Ca^{2+} uptake and catecholamine secretion

Cholinergic agonist-stimulated Ca^{2+} uptake and secretion were compared pharmacologically. In previous studies it had been demonstrated that catecholamine secretion resulted from stimulation

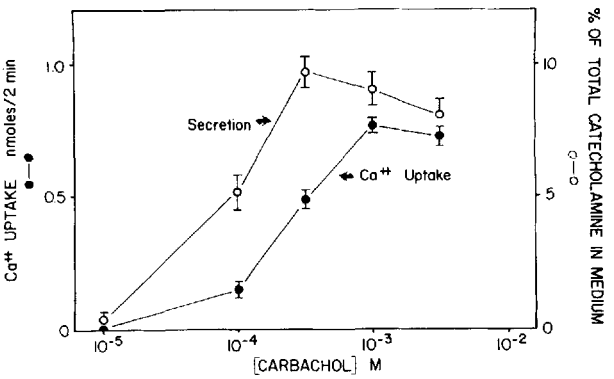

FIG. 2. Carbachol dose-response curve for Ca^{2+} uptake and catecholamine secretion. Chromaffin cells were incubated in various concentrations of carbachol in $^{45}Ca^{2+}$-containing PSS. Ca^{2+} uptake and catecholamine secretion were determined after 2 min. There were four wells per group.

of a nicotinic receptor (Mizobe et al., 1979; Kilpatrick et al., 1980; Fisher et al., 1981). The pharmacology of Ca^{2+} uptake was determined using both a series of nicotinic and muscarinic agonists and specific cholinergic antagonists. Ca^{2+} uptake was induced by the nicotinic agonists nicotine and DMPP and the mixed nicotinic-muscarinic agonists carbachol and acetylcholine (Fig. 3). In contrast, the muscarinic agonists muscarine and methacholine did not stimulate Ca^{2+} uptake. The relatively small amount of nicotine-induced Ca^{2+} uptake (in 2 min) correlated with the relatively low initial rate of nicotine-induced catecholamine secretion compared with carbachol- or acetylcholine-induced secretion (data not shown). Mecamylamine (a nicotinic antagonist), but not atropine (a muscarinic antagonist), blocked carbachol-induced catecholamine secretion and Ca^{2+} uptake (Fig. 4). Tubocurarine, another nicotinic antagonist, also specifically blocked Ca^{2+} uptake and secretion (data not shown). Thus, both Ca^{2+} uptake and catecholamine secretion were induced by activation of a nicotinic receptor. The pharmacological specificity also strongly suggests that Ca^{2+} uptake occurred in chromaffin cells and not in other cell types contaminating the cultures (Kilpatrick et al., 1980).

Depolarization-induced secretion and Ca^{2+} uptake

The relationship between catecholamine secretion and Ca^{2+} uptake was investigated in cells incubated in depolarizing concentrations of K^+ (56 m*M*). Secretion was approximately linear with time for 2 min and then slowed, but did not cease, from 2 to 15 min (Fig. 5A). At 15 s, elevated K^+-induced Ca^{2+} uptake was 30% of maximal, whereas catecholamine secretion was barely detectable (Fig. 5B). Apparent Ca^{2+} uptake was maximal during the 1st min, and, thereafter, slowed and then stopped. Although continued secretion between 5 and 15 min was not pronounced (Fig. 5A), in experiments with other cell preparations continued secretion was clearly evident at these times and was associated with a small but significant Ca^{2+} uptake.

In experiments with elevated K^+, the NaCl concentration of the medium was reduced from 142 m*M* to 92 m*M*. To determine whether the effects of solutions containing elevated K^+ were specifically caused by the elevated K^+ and not by the reduced Na^+, the effects of various other cation substitutes for Na^+ were examined for their ability to stimulate Ca^{2+} uptake and secretion (Table 2). Li^+, $Tris^+$, and $choline^+$ all failed to stimulate Ca^{2+} uptake and secretion. The effects of K^+ were, therefore, specific, and were caused by the depolarizing action of K^+, rather than by the reduction of Na^+ in the medium.

Effects of varying Ca^{2+} concentrations on Ca^{2+} uptake and catecholamine secretion

The relationship between medium Ca^{2+} concentration and Ca^{2+} uptake and catecholamine secretion were examined in cells incubated with either carbachol or 56 m*M* K^+. Carbachol (0.3 m*M*) or elevated K^+ induced at least a twofold increase in Ca^{2+} uptake at all Ca^{2+} concentrations. Carbachol-induced Ca^{2+} uptake was linear between 0 and 1 min from 0.1 to 2.2 m*M* Ca^{2+}, but was sublinear at 15 m*M* Ca^{2+}. Elevated K^+-induced Ca^{2+} uptake was sublinear by 1 min at all Ca^{2+} concentrations examined (0.1–15 m*M*). Figure 6, A and B, shows comparative time courses at 2.2 m*M* and 15 m*M* Ca^{2+}.

The Ca^{2+} dependency of Ca^{2+} uptake was qual-

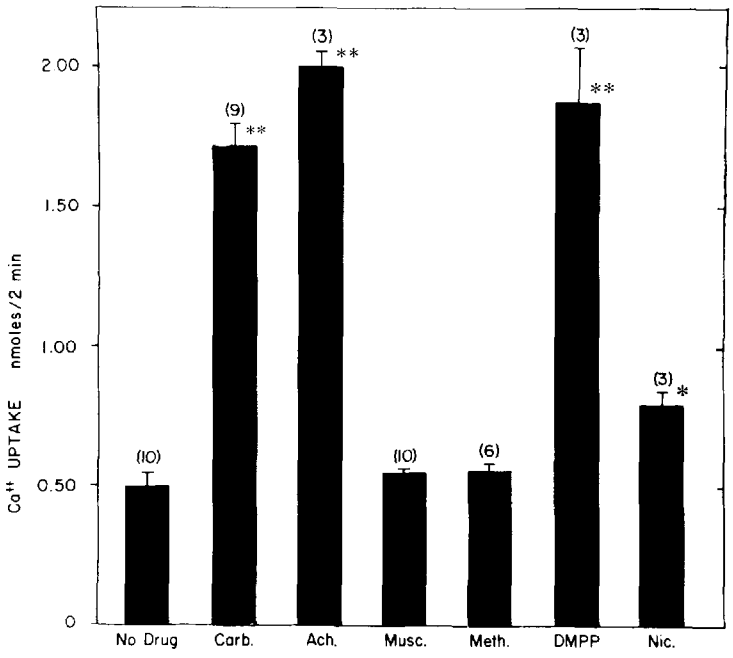

FIG. 3. Effects of various cholinergic agonists on Ca^{2+} uptake into chromaffin cells. Chromaffin cells were incubated for 2 min in $^{45}Ca^{2+}$-containing solution in the presence of no drug, 0.3 m*M* carbachol (Carb.), 0.3 m*M* acetylcholine (Ach.), 0.3 m*M* muscarine (Musc.), 0.3 m*M* methacholine (Meth.), 10 μM 1,1-dimethyl-4-phenylpiperazinium (DMPP), or 10 μM nicotine (Nic.). Ca^{2+} uptake was determined as described in Materials and Methods. The numbers in parentheses above the error bars denote the number of wells per group. Single asterisk denotes $p < 0.05$ versus no drug; double asterisk $p < 0.001$ versus no drug.

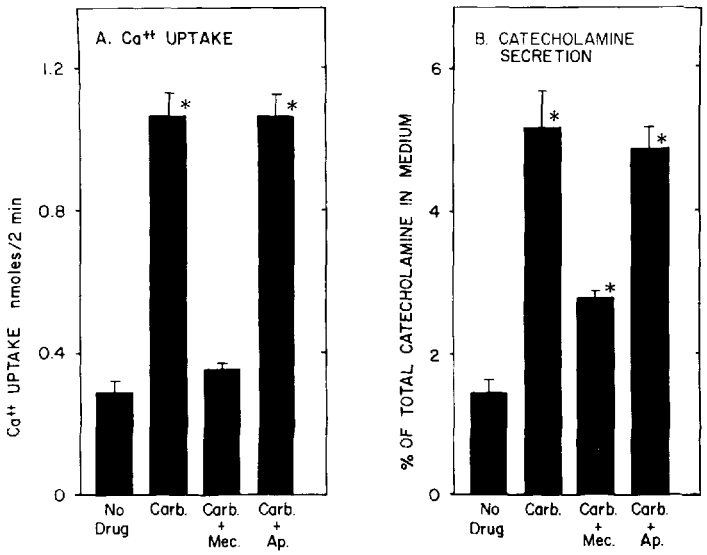

FIG. 4. Effects of cholinergic antagonists on carbachol-induced catecholamine secretion and Ca^{2+} uptake. Chromaffin cells were incubated in the absence of drug or in the presence of 0.3 m*M* carbachol (Carb.) with or without 2 μM mecamylamine (Mec.) or 2 μM atropine (Ap.). After 2 min in $^{45}Ca^{2+}$-containing solution **(A)** or nonradioactive solution **(B)** Ca^{2+} uptake or catecholamine secretion was measured as described in Materials and Methods. Cells incubated in the presence of antagonist and carbachol had been preincubated for 15 min in solution containing antagonist alone. Mecamylamine (2 μM) or atropine (2 μM) alone did not alter Ca^{2+} uptake or catecholamine secretion compared with the absence of drug. There were four wells per group. Asterisk denotes $p < 0.001$ versus no drug.

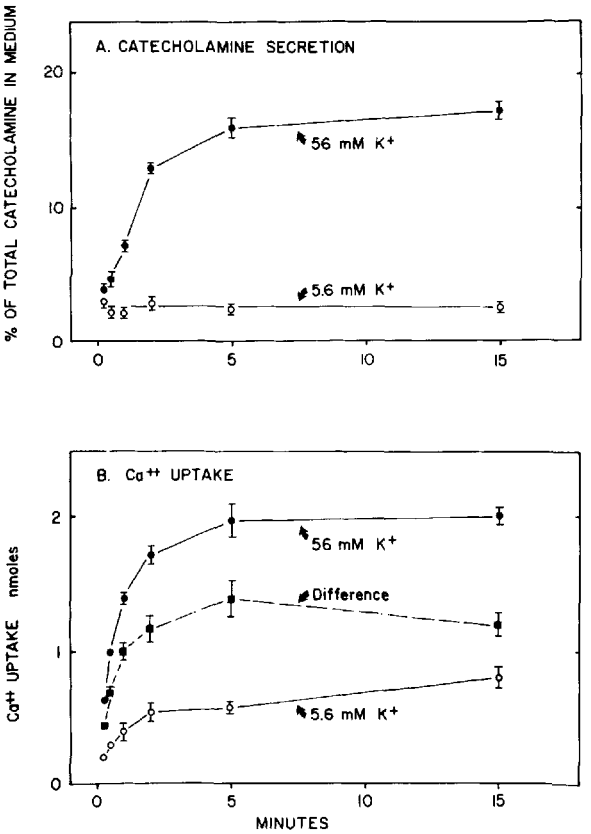

FIG. 5. Time course of depolarization-induced catecholamine secretion **(A)** and Ca^{2+} uptake **(B)**. Cells were incubated in solution containing $^{45}Ca^{2+}$ and either 5.6 m*M* or 56 m*M* K^+. Catecholamine secretion and Ca^{2+} uptake into cells were determined after various incubation times. The dashed line in B represents the difference between Ca^{2+} uptake in 56 and 5.6 m*M* K^+ (the depolarization-induced Ca^{2+} uptake). There were four wells per group. The standard error of the mean bars of some points were smaller than the point symbols and were omitted.

itatively different for carbachol and elevated K^+. Carbachol-induced Ca^{2+} uptake increased with Ca^{2+} concentration from 0.1 to 15 m*M* Ca^{2+}, whereas elevated K^+-induced Ca^{2+} uptake was maximal between 2 m*M* and 7 m*M* Ca^{2+} and then declined at 15 m*M* Ca^{2+} (Fig. 6C). A nonlinear regression analysis of carbachol-induced Ca^{2+} uptake between 0.1 m*M* and 2.2 m*M* Ca^{2+} gave an apparent K_m of 2 m*M*. Estimation of an apparent K_m for elevated K^+-induced Ca^{2+} uptake was complicated by our inability to measure an initial rate of Ca^{2+} uptake at 1 min and by the inhibition of Ca^{2+} uptake at high Ca^{2+} concentrations. However, because the degree of sublinearity was independent of Ca^{2+} concentration, Ca^{2+} uptake at low Ca^{2+} concentrations (0.1–1.0 m*M*) was subjected to a nonlinear regression analysis and yielded values for an apparent K_m of 1.4–2 m*M*.

TABLE 2. *Effects of various Na^+ substitutes on Ca^{2+} uptake and catecholamine secretion*

Substitution	Ca^{2+} uptake (nmol/2 min)	Catecholamine secretion (% of catecholamine in 2 min)
None	0.51 ± 0.05	3.2 ± 0.2
K^+	1.83 ± 0.05[a]	5.9 ± 0.3[a]
Li^+	0.61 ± 0.02	2.6 ± 0.2
$Tris^+$	0.49 ± 0.01	2.1 ± 0.2
$Choline^+$ (+ 50 μM mecamylamine)	0.54 ± 0.02	3.0 ± 0.1

PSS was prepared with either a standard concentration of NaCl (142 m*M*) or with 92 m*M* NaCl and an additional 50 m*M* of either KCl, LiCl, Tris chloride, or choline chloride. The osmolality of all solutions was 310–315 mOs and the pH was 7.4. The choline-containing solution contained 50 μM mecamylamine to block nicotinic effects of choline (Holz and Senter, 1981). Ca^{2+} uptake and secretion during a 2-min incubation were determined. There were four wells per group.

[a] $p < 0.001$ versus no substitution.

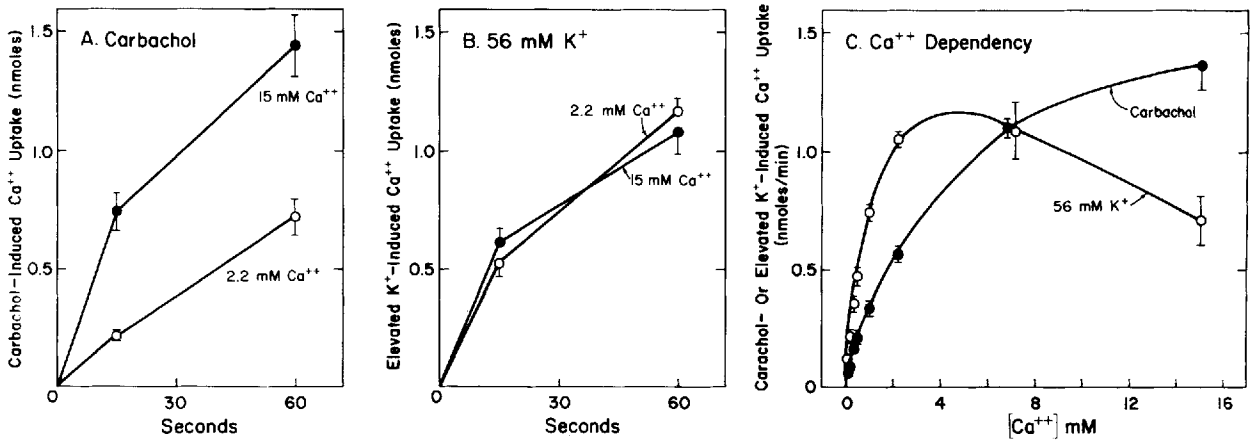

FIG. 6. Effect of varying the Ca^{2+} concentration on carbachol- and elevated K^+-induced Ca^{2+} uptake. Cells were washed with 1 ml PSS containing 0.5 m*M* $MgCl_2$ and various concentrations of $CaCl_2$. The wash solution was removed, and test solution containing the same $CaCl_2$ concentration, 0.5 m*M* $MgCl_2$, and no other addition, 0.3 m*M* carbachol, or 56 m*M* K^+ was added to the cells. The difference in Ca^{2+} uptake in the presence and absence of carbachol or elevated K^+ was calculated. There were four wells per group. Carbachol-induced **(A)** or elevated K^+-induced **(B)** Ca^{2+} uptake was measured after 15 s or 60 s in the presence of 2.2 or 15 m*M* Ca^{2+}. **(C)** Carbachol- or elevated K^+-induced Ca^{2+} uptake was measured after 1 min stimulation in various concentrations of Ca^{2+}.

Because carbachol- or elevated K^+-induced secretion of catecholamine after 1 minute was only 10–70% that of the background catecholamine in the medium, a quantitative correlation between Ca^{2+} uptake and catecholamine secretion after a 1-min exposure to the secretagogue was not feasible. However, at 2 min catecholamine secretion and Ca^{2+} uptake saturated over the same Ca^{2+} concentration range with either carbachol or elevated K^+ stimulation (Fig. 7).

Effects of various divalent ion inhibitors on Ca^{2+} uptake and catecholamine secretion

Mg^{2+} inhibits Ca^{2+}-induced secretion in a number of secretory systems, including the perfused adrenal medulla (Douglas and Rubin, 1964*b*). In the present system, Mg^{2+} at concentrations of 2 m*M* or more inhibited depolarization-induced catecholamine secretion (Fig. 8A) and Ca^{2+} uptake (Fig. 8B). Cd^{2+} and Zn^{2+} also inhibit Ca^{2+} effects in a variety of systems (Akaike et al., 1978; Benoit and Manbrini,

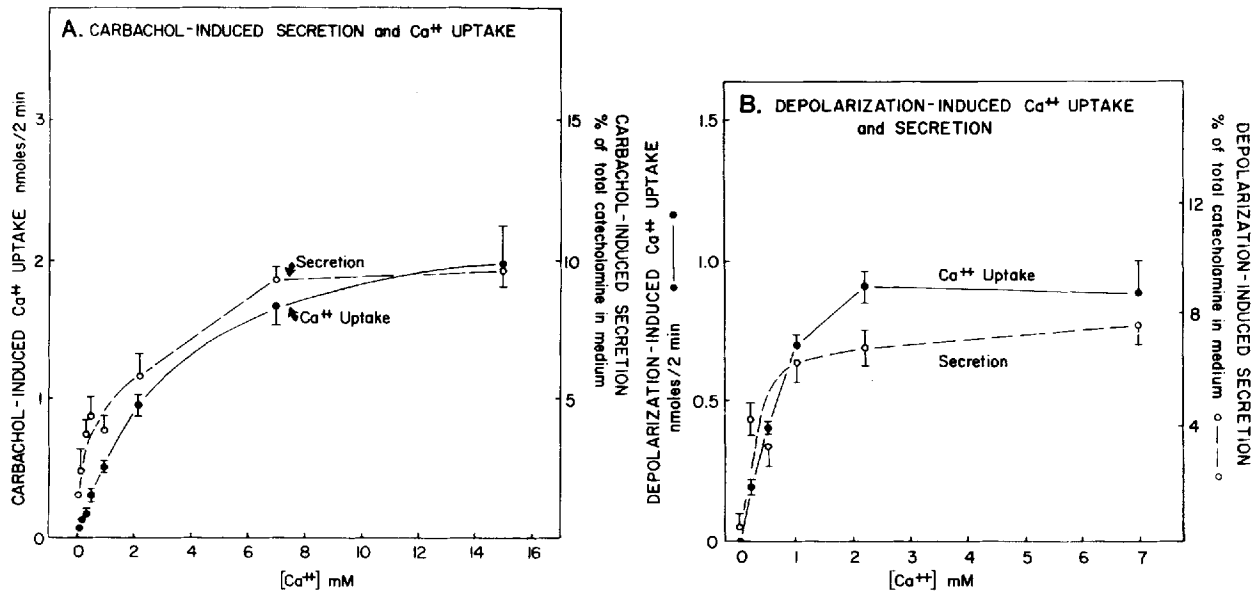

FIG. 7. Correlation between catecholamine secretion and Ca^{2+} uptake at different Ca^{2+} concentrations. Cells were washed with 1 ml PSS containing 0.5 m*M* $MgCl_2$ and various concentrations of $CaCl_2$. The wash solution was removed and test solution containing the same $CaCl_2$ concentration, 0.5 m*M* $MgCl_2$, and no other addition, 0.3 m*M* carbachol, or 56 m*M* K^+ was added to the cells. Ca^{2+} uptake and catecholamine secretion were determined after 2 min. The secretagogue-induced Ca^{2+} uptake or catecholamine secretion was calculated. There were four wells per group.

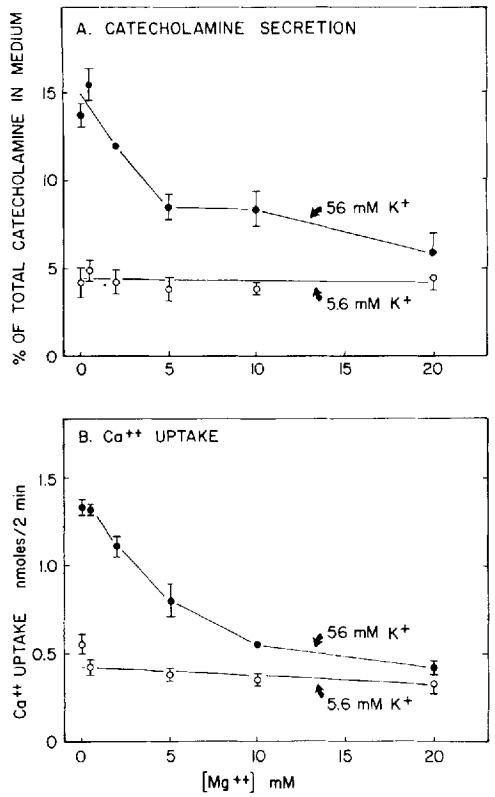

FIG. 8. Effects of Mg^{2+} on depolarization-induced catecholamine secretion **(A)** and Ca^{2+} uptake **(B)**. Cells were incubated for 2 min in a solution containing 2.2 m*M* Ca^{2+} and either 5.6 or 56 m*M* K^+. Magnesium chloride concentrations were varied as indicated. Catecholamine secretion and Ca^{2+} uptake were measured in parallel experiments. There were four wells per group.

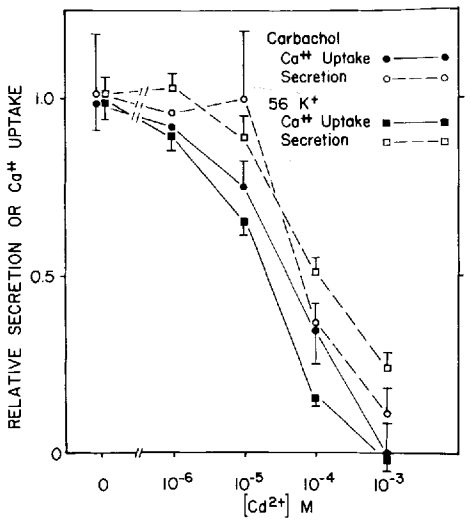

FIG. 9. The effect of Cd^{2+} on carbachol- and elevated K^+-induced Ca^{2+} uptake and catecholamine secretion. Chromaffin cells were incubated in the presence or absence of carbachol or 56 m*M* K^+. Also present were various concentrations of Cd^{2+}. Ca^{2+} uptake and catecholamine secretion in a 2-min incubation were determined. Data are expressed as the fraction of carbachol-induced or elevated K^+-induced secretion in the absence of Cd^{2+}. Background Ca^{2+} uptake or catecholamine release in the absence of secretagogue was subtracted. There were four wells per group.

1970). Cd^{2+} ($10^{-5}-10^{-4}$ *M*) strongly inhibited Ca^{2+} uptake and catecholamine secretion induced by either carbachol or 56 m*M* K^+ (Fig. 9). Zn^{2+} (1 m*M*) inhibited elevated K^+-stimulated Ca^{2+} uptake and catecholamine secretion by over 80% (data not shown). These data with various divalent ion antagonists further demonstrate a correlation between Ca^{2+} uptake and catecholamine secretion.

Ba^{2+} in the absence of Ca^{2+} or other secretagogues initiates secretion from chromaffin cells (Douglas and Rubin, 1964*a*). Ba^{2+} probably acts after entering the cell. Because Cd^{2+} (10^{-5} *M*) inhibited Ba^{2+}-induced secretion (Table 3), it is likely that Cd^{2+} blocks Ba^{2+}, as well as Ca^{2+}, entry.

Inactivation of carbachol- and 56 m*M* K^+-induced Ca^{2+} uptake and catecholamine secretion

To determine whether the decreased rate of Ca^{2+} uptake and catecholamine secretion after a 15-min exposure to carbachol required extracellular Ca^{2+}, cells were preincubated for 15 min, in the absence of Ca^{2+}, in 0.3 m*M* carbachol. In the absence of Ca^{2+}, carbachol-induced secretion did not occur (see Fig. 10, legend). A solution containing both 2.2 m*M* Ca^{2+} and 0.3 m*M* carbachol was then added, and Ca^{2+} uptake and secretion were measured in the subsequent 2-min period (Fig. 10). Preincubation in 0.3 m*M* carbachol in the absence of Ca^{2+} inhibited subsequent carbachol-induced Ca^{2+} uptake 49%. Catecholamine secretion was not inhibited. Preincubation for 15 min in 0.3 m*M* carbachol in the presence of 2.2 m*M* Ca^{2+} inhibited subsequent carbachol-induced Ca^{2+} uptake and catecholamine secretion 92% and 86%, respectively. In three other experiments virtually identical results were obtained, except in one, in which a small (28%) but statistically significant inhibition of carbachol- and Ca^{2+}-dependent catecholamine secretion was observed after preincubation without Ca^{2+} in the presence of carbachol. Hence, desensitization, both to

TABLE 3. *Effects of Cd^{2+} on Ba^{2+}-induced secretion*

Additional divalent ions	% of total catecholamine in medium
None	3.2 ± 0.2
Ca^{2+} (2.2 m*M*)	1.5 ± 0.1
Ba^{2+} (2.2 m*M*)	41.0 ± 0.9[a]
Ba^{2+} (2.2 m*M*) + Cd^{2+} (10^{-5} *M*)	22.3 ± 0.7[a]
Ba^{2+} (2.2 m*M*) + Cd^{2+} (10^{-4} *M*)	1.5 ± 0.3

Monolayers were washed with 1 ml Ca^{2+}-free PSS immediately before the test solution was added. All solutions contained 0.5 m*M* $MgCl_2$. There were four wells per group.

[a] $p < 0.001$ versus solution with no additional divalent ions.

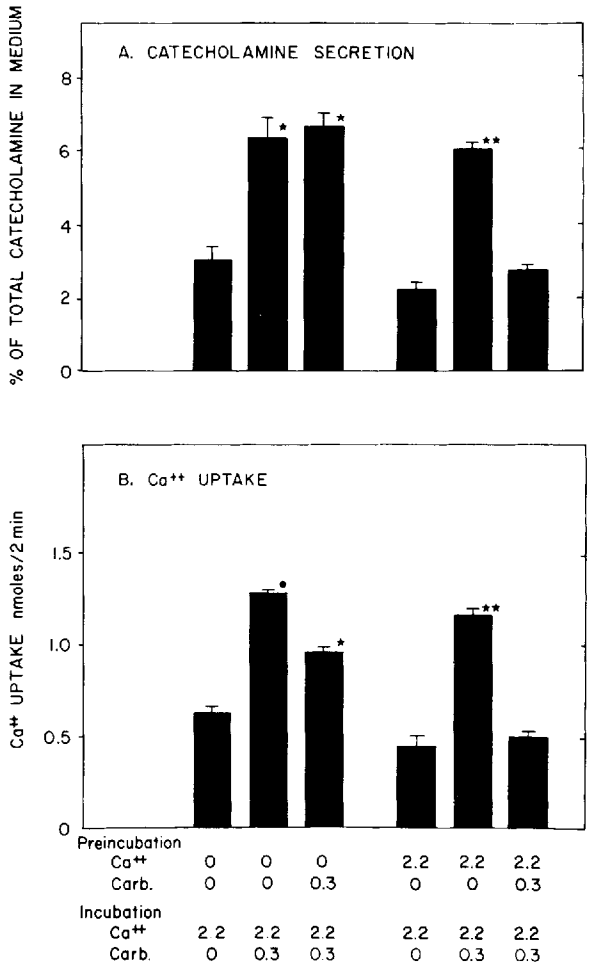

FIG. 10. Effects of Ca^{2+} on the inactivation of carbachol-induced secretion and Ca^{2+} uptake. Cells were preincubated in PSS containing 0 or 2.2 m*M* Ca^{2+} in the presence or absence of 0.3 m*M* carbachol. After 15 min the preincubation medium was aspirated and incubation medium containing 2.2 m*M* $^{45}Ca^{2+}$ and either 0 or 0.3 m*M* carbachol was added. Catecholamine secretion **(A)** and Ca^{2+} uptake **(B)** were determined during a 2-min incubation. All solutions contained 0.5 m*M* $MgCl_2$. Carbachol-induced secretion during the 15-min preincubation in the absence of Ca^{2+} was less than 1% and in the presence of Ca^{2+} was 9.8 ± 0.4%. Secretion data in the graph were expressed as percentage of total catecholamine present in wells when the preincubation solution was added. There were four wells per group. Single asterisk denotes $p < 0.001$ versus 0 Ca^{2+} and 0 carbachol in the preincubation medium and 2.2 m*M* Ca^{2+} and 0 carbachol in the incubation medium. Double asterisk denotes $p < 0.001$ versus 2.2 Ca^{2+} and 0 carbachol in both the preincubation and incubation media.

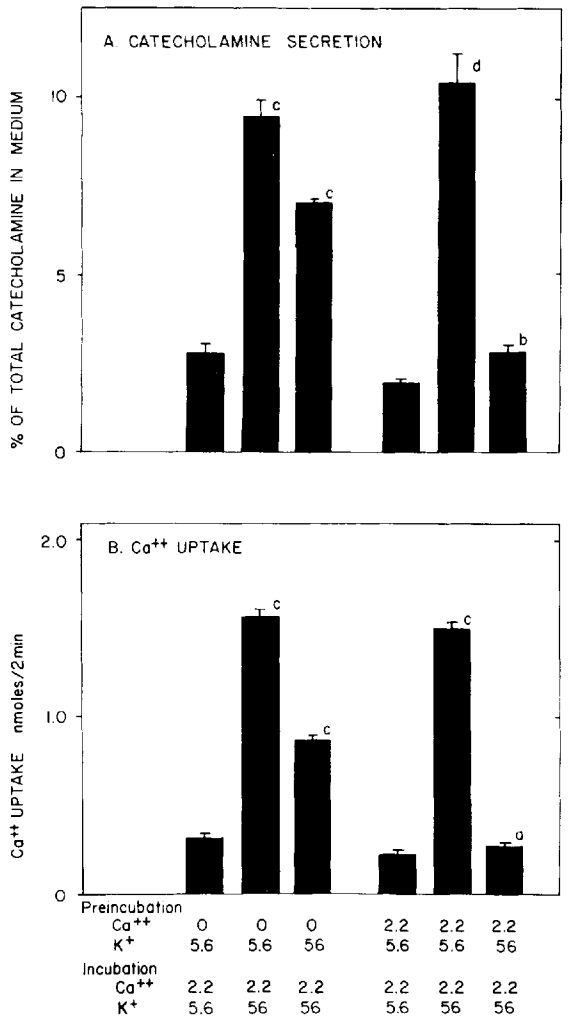

FIG. 11. Effects of Ca^{2+} on the inactivation of depolarization-induced secretion **(A)** and Ca^{2+} uptake **(B).** Cells were preincubated in either 5.6 or 56 m*M* K^+-containing solution with or without 2.2 m*M* Ca^{2+}. After 15 min the preincubation medium was aspirated and incubation medium was added containing 2.2 m*M* Ca^{2+} and either 5.6 or 56 m*M* K^+. Catecholamine secretion and Ca^{2+} uptake were determined after 2 min in parallel experiments. When Ca^{2+} uptake was measured, the incubation medium also contained $^{45}Ca^{2+}$. The percentage of the total catecholamine released into the medium after preincubation in 2.2 m*M* Ca^{2+} and 56 m*M* K^+ was 15.4 ± 0.1. In all other groups 2–3% of the total catecholamine was released during the preincubation. Catecholamine in the medium is expressed as the percentage of total catecholamine present in wells when the preincubation solution was added. There were four wells per group. Significance (a–d): a: $p < 0.02$ versus preincubation and incubation in 2.2 m*M* Ca^{2+} and 5.6 m*M* K^+. b: $p < 0.01$ versus preincubation and incubation in 2.2 m*M* Ca^{2+} and 5.6 m*M* K^+. c: $p < 0.001$ versus preincubation in 0 Ca^{2+} and 5.6 m*M* K^+ and incubation in 2.2 m*M* Ca^{2+} and 5.6 m*M* K^+. d: $p < 0.001$ versus preincubation and incubation in 2.2 m*M* Ca^{2+} and 5.6 m*M* K^+.

carbachol-induced Ca^{2+} uptake and to carbachol-induced catecholamine secretion, was enhanced by—or required—the presence of Ca^{2+} in the medium.

The effect of extracellular Ca^{2+} on the time course of depolarization-induced Ca^{2+} uptake and catecholamine secretion was also investigated (Fig. 11). Cells were preincubated for 15 min in the presence or absence of Ca^{2+} in 5.6 or 56 m*M* K^+. A solution containing Ca^{2+} and either 5.6 or 56 m*M* K^+ was then added; both secretion and Ca^{2+} uptake were measured in the subsequent 2-min period. Preincubation in elevated K^+ in the absence of Ca^{2+} inhibited secretion and Ca^{2+} uptake 35% and 55%,

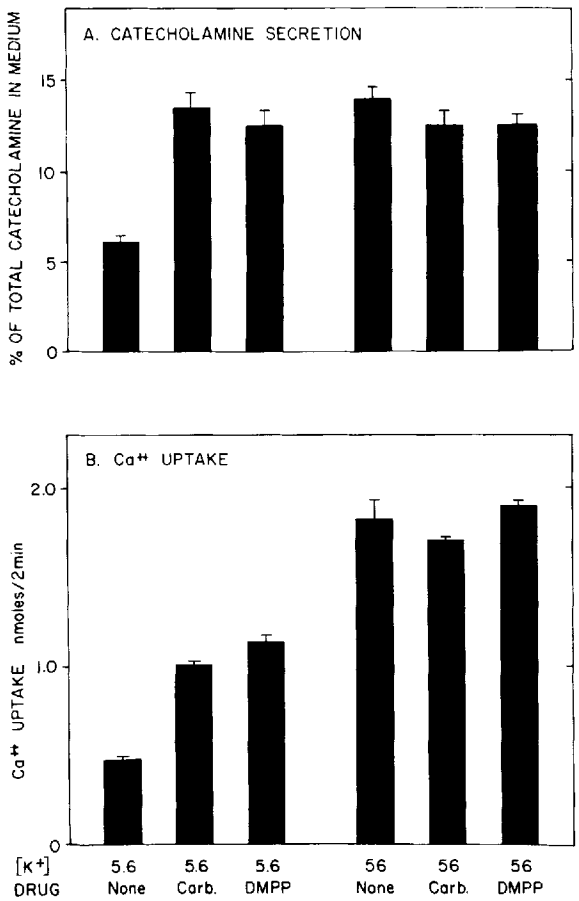

FIG. 12. Effects of elevated K^+ on nicotinic stimulation of catecholamine secretion **(A)** and Ca^{2+} uptake **(B).** Catecholamine secretion and Ca^{2+} uptake were determined during a 2-min incubation of cells in PSS containing either 5.6 or 56 m*M* K^+ in the presence or absence of various drugs. None = no drug; Carb. = 0.3 m*M* carbachol; DMPP = 10 μM 1,1-dimethyl-4-phenylpiperazinium. There were six samples per group. For both catecholamine secretion and Ca^{2+} uptake all groups were significantly different ($p < 0.001$) from catecholamine secretion and Ca^{2+} uptake, respectively, in cells incubated in 5.6 m*M* K^+ in the absence of drug.

respectively. In contrast, preincubation in elevated K^+ in the presence of Ca^{2+} inhibited secretion and Ca^{2+} uptake 89% and 96%, respectively. Hence, both secretion and Ca^{2+} uptake were significantly more inhibited when the depolarizing preincubation medium contained Ca^{2+}. It is unlikely that the increased inhibition after exposure to elevated K^+ in the presence of Ca^{2+} was related to the approximately 15% depletion of catecholamine and a (presumed) similar proportional decrease in the number of secretory vesicles. Cells exposed for 15 min to solution containing 56 m*M* K^+ and 2.2 m*M* Ca^{2+} and incubated for 1 h at 25°C in PSS containing 2.2 m*M* Ca^{2+} and 5.6 m*M* K^+ remained depleted of catecholamine. Depolarization-induced Ca^{2+} uptake and catecholamine secretion were inhibited 21% and 31%, respectively, indicating that secretion and Ca^{2+} uptake had partially recovered after the preincubation. Thus, catecholamine depletion alone cannot completely account for the inhibition of Ca^{2+} uptake and secretion after exposure to elevated K^+ in the presence of Ca^{2+}.

Effects of elevated K^+ on nicotinic stimulation of catecholamine secretion and Ca^{2+} uptake

To investigate whether nicotinic agonist-induced effects and depolarization-induced effects can occur simultaneously and independently, catecholamine secretion and Ca^{2+} uptake were determined in cells incubated in either 5.6 m*M* or 56 m*M* K^+ in the presence or absence of carbachol or DMPP (Fig. 12). Neither carbachol nor DMPP altered catecholamine secretion or Ca^{2+} uptake in the presence of 56 m*M* K^+. In another experiment (data not shown), cells were preincubated for 15 min in the presence of 56 m*M* K^+ and 2.2 m*M* Ca^{2+}, by which time depolarization-induced Ca^{2+} uptake and catecholamine secretion had virtually ceased. The subsequent addition of 10 μM DMPP, in the continued presence of 56 m*M* K^+ and 2.2 m*M* Ca^{2+}, stimulated neither Ca^{2+} uptake nor catecholamine secretion.

Ca^{2+} content of cells

In the above experiments, Ca^{2+} uptake from the medium was determined by measuring the influx of $^{45}Ca^{2+}$ from the medium. This uptake could represent Ca^{2+}-Ca^{2+} exchange across the plasma membrane or net Ca^{2+} uptake. To assess net Ca^{2+} uptake directly, cells were incubated for 2 min in solution containing either 5.6 or 56 m*M* K^+. The cells were washed three times with Ca^{2+}-free PSS containing 0.5 m*M* Mg^{2+}. The Ca^{2+} content of the cells were measured by atomic absorption spectroscopy. The Ca^{2+} contents of cells incubated in 5.6 m*M* K^+ and 56 m*M* K^+ were 4.98 ± 0.76 ($n = 3$) and 5.51 ± 0.29 ($n = 4$) nmol/well, respectively. These values were not significantly different from each other. Based upon the $^{45}Ca^{2+}$ measurements, we expected net uptake to be no greater than 1 nmol/well, which could not be readily detected because of the high and somewhat variable Ca^{2+} content of the wells. Thus, the possibility exists that an unknown fraction of the measured $^{45}Ca^{2+}$ uptake represents Ca^{2+}-Ca^{2+} exchange and not net Ca^{2+} uptake. However, because of the generally close correlation between Ca^{2+} uptake as measured by $^{45}Ca^{2+}$ influx and catecholamine secretion (see Discussion), and because of the absolute dependency of secretion on extracellular Ca^{2+}, it is likely that a substantial fraction of the Ca^{2+} influx measured by $^{45}Ca^{2+}$ represents net uptake.

DISCUSSION

Relationship between Ca^{2+} uptake and secretion

The development of techniques to maintain and investigate primary dissociated cultures of chro-

maffin cells from bovine adrenal medulla allows one to investigate the relationship between Ca^{2+} uptake and exocytotic release of catecholamines, with a time resolution of 10–15 s. In previous studies with either perfused adrenal medulla (Douglas and Poisner, 1961) or adrenal medullary slices (Baker and Rink, 1975) time resolution was considerably less, and it has been difficult to make precise quantitative comparisons between different experimental groups. Baker and Rink (1975) observed that in adrenal medullary slices $^{45}Ca^{2+}$ uptake continued after prolonged exposure to elevated K^+ at a time when catecholamine secretion had returned to baseline. In the present study we found a close kinetic and pharmacological correlation between $^{45}Ca^{2+}$ uptake and catecholamine, as follows.

1. The cholinergic agonist carbachol and a depolarizing concentration of K^+ stimulated significant Ca^{2+} uptake within 15 s, before catecholamine secretion was readily detectable. At later times the kinetics of secretion induced by carbachol or elevated K^+ were similar to that of Ca^{2+} uptake.

2. Pharmacological experiments with cholinergic agonists and antagonists demonstrated that a nicotinic receptor was responsible for both cholinergic activation of Ca^{2+} uptake and catecholamine secretion.

3. Ca^{2+} uptake and catecholamine secretion induced by carbachol saturated over an identical Ca^{2+} concentration range. Similar results were obtained with stimulation by elevated K^+.

4. Inhibition of Ca^{2+} uptake and catecholamine secretion occurred over similar concentration ranges of Mg^{2+} and Cd^{2+}.

Thus, the present study strongly supports the conclusion from the now classical work of Douglas and co-workers (Douglas, 1975) that Ca^{2+} entry initiates and maintains secretion from adrenal medullary cells.

Ca^{2+} dependency of carbachol-induced Ca^{2+} uptake

Ca^{2+} uptake induced by carbachol saturated as the extracellular Ca^{2+} concentration increased, with an apparent K_m of approximately 2 mM. It is unlikely that the saturation of Ca^{2+} uptake resulted from saturation of intracellular Ca^{2+} sequestration sites, since this would tend to increase the cytosolic Ca^{2+} concentration and, thereby, increase catecholamine secretion. Because changes in catecholamine secretion paralleled changes in Ca^{2+} uptake, it is more likely that the saturation of Ca^{2+} uptake reflected an effect of Ca^{2+} on Ca^{2+} transport into the cells. It is tempting to ascribe the saturation of Ca^{2+} uptake to saturable binding of Ca^{2+} to a site on the nicotinic agonist-activated ionic channel; however, the saturation may be at least partially caused by a Ca^{2+}-induced increase in the rate of nicotinic channel desensitization. Indeed, the presence of Ca^{2+} during a 15-min exposure of cells to carbachol greatly enhanced the degree of inactivation of Ca^{2+} uptake and catecholamine secretion during the subsequent 2 min (Fig. 10). The sublinearity of the time course of Ca^{2+} uptake caused by elevated Ca^{2+} is also consistent with a Ca^{2+}-induced increase in the rate of desensitization. The ability of Ca^{2+} to increase the rate of nicotinic receptor desensitization has been previously demonstrated with the frog neuromuscular junction (Manthey, 1966; Magazanik and Vyskočil, 1970). Ca^{2+} also shortens the nicotinic channel open time on frog skeletal muscle (Bregestovski et al., 1979).

Inactivation by Ca^{2+} of voltage-sensitive Ca^{2+} channels

Elevated K^+ (56 mM) caused a rapid Ca^{2+} influx in the first 15 s, which subsequently slowed. A similar effect had been observed in perfused adrenal medulla and in adrenal medullary slices (Douglas and Rubin, 1963; Baker and Rink, 1975). Inactivation of Ca^{2+} entry was reduced if depolarization occurred in the absence of Ca^{2+}. Ca^{2+} entry via voltage-sensitive Ca^{2+} channels may have not only initiated secretion but also inactivated these same channels. Consistent with this hypothesis is the Ca^{2+} dependency of Ca^{2+} entry through the voltage-sensitive channels. Increasing the extracellular Ca^{2+} concentration from 7 to 15 mM reduced the absolute Ca^{2+} uptake. These results contrast with those obtained with carbachol, in which Ca^{2+} uptake increased between 0.1 and 15 mM Ca^{2+}. If Ca^{2+} entry is responsible for part of the inactivation of the voltage-sensitive Ca^{2+} permeability, the Ca^{2+}-dependent inactivation process must occur within 15 s, since it does not alter the time course between 15 s and 60 s (Fig. 6B). Although the data from a variety of different experiments are consistent with Ca^{2+}-induced inactivation of voltage-sensitive Ca^{2+} channels, a direct demonstration must await an appropriate electrophysiological study. To our knowledge, Ca^{2+}-dependent inactivation of Ca^{2+} channels has not been previously demonstrated in mammalian systems, although it has been extensively investigated in paramecium (Brehm and Eckert, 1978), snail neurons (Kostyuk and Krishtal, 1977; Standen, 1981), insect muscle (Ashcroft and Stanfield, 1981), and Aplysia (Tillotson, 1979).

Depolarization-induced inactivation of Ca^{2+} uptake in the absence of Ca^{2+} was also observed. It may represent a voltage-dependent inactivation of Ca^{2+} channels.

Evidence for saturable intracellular Ca^{2+}-dependent processes

Although there was generally an excellent correlation between Ca^{2+} uptake and catecholamine secretion, there were also quantitative differences in the responses of both processes in various experiments. The correlations were best when stimulated

Ca^{2+} uptake was small. When stimulated Ca^{2+} uptake was large, changes in Ca^{2+} uptake resulted in a less than proportional change in catecholamine secretion, as in the following examples.

1. In the carbachol dose-response experiment (Fig. 2), Ca^{2+} uptake continued to increase above 0.3 m*M* carbachol, although secretion did not increase.

2. The lowest concentration of Cd^{2+} necessary for inhibition of both carbachol- and elevated K^+-stimulated Ca^{2+} uptake was significantly lower than that required to begin to inhibit catecholamine secretion (Fig. 9).

3. Preincubation in carbachol in the absence of Ca^{2+} inhibited subsequent carbachol-induced Ca^{2+}, uptake with little or no alteration of secretion (Fig. 10 and text).

Because there are contaminating non-chromaffin cells in the culture (Kilpatrick et al., 1980) it is possible that some of the lack of precise correlation of Ca^{2+} uptake with catecholamine secretion was caused by uptake into non-chromaffin cells. Depolarization induced by elevated K^+ may have stimulated Ca^{2+} uptake into non-chromaffin cells containing voltage-sensitive Ca^{2+} channels. However, the strict nicotinic specificity of cholinergic stimulation of both Ca^{2+} uptake and catecholamine secretion suggests that chromaffin cells were mainly responsible for cholinergic-induced Ca^{2+} uptake. An explanation consistent with the data is that elevated cytosolic Ca^{2+} saturated intracellular Ca^{2+}-dependent reactions which cause exocytosis. It is also possible that cytosolic Ca^{2+} concentrations rose less than proportionally with $^{45}Ca^{2+}$ uptake.

Lack of additivity of nicotinic agonist-induced effects and depolarization-induced effects

If nicotinic stimulation occurs independently of stimulation induced by elevated K^+, then catecholamine secretion and Ca^{2+} uptake in the presence of both stimuli together should be additive or at least greater than either alone. Instead, nicotinic stimulation had no additive effect on either Ca^{2+} uptake or catecholamine secretion in the presence of 56 m*M* K^+. Lack of effect on secretion of nicotinic stimulation in the presence of elevated K^+ had been observed previously (Kilpatrick et al., 1981). It is possible that both nicotinic stimulation and elevated K^+ have a common mechanism, depolarization, that activates voltage-sensitive Ca^{2+} channels, thereby initiating Ca^{2+} uptake and catecholamine secretion. Acetylcholine depolarizes adrenal medullary cells (Douglas et al., 1967; Brandt et al., 1976; Biales et al., 1976), however, in the present study the different Ca^{2+} dependencies of Ca^{2+} uptake induced by carbachol and elevated K^+ suggest that the Ca^{2+} permeability mechanisms are different for the two stimuli. Furthermore, Kilpatrick et al. (1981) demonstrated that in cultured chromaffin cells acetylcholine stimulated vigorous secretion in media that prevent acetylcholine-induced depolarization (sucrose substituted isoosmotically for NaCl). A more likely explanation for the effect of nicotinic stimulation on chromaffin cells is that the ionic channel, which is directly coupled to the nicotinic receptor, allowed Ca^{2+} entry. Kidokoro and Ritchie (1980) also suggested that Ca^{2+} entry in rat adrenal medullary cells in the presence of high concentrations of acetylcholine occurs mainly through acetylcholine receptor channels. Depolarization and Ca^{2+} entry may inhibit the function of the nicotinic receptor channel. Indeed, the nicotinic channel on frog skeletal muscle is permeable to Ca^{2+} (Bregestovski et al., 1979), and its open time is reduced by depolarization (Anderson and Stevens, 1973) and possibly by Ca^{2+} entry (Bregestovski et al., 1979). The large depolarization induced by 56 m*M* K^+ in the present studies may have reduced the open time of nicotinic receptor-coupled channels, so that no detectable nicotinic stimulation occurred. Elevated K^+-induced calcium entry may have also reduced the channel open time.

Role of intracellular Ca^{2+} in exocytosis

An issue of fundamental importance, which the current study did not directly address, was the nature of events within the cell, initiated by Ca^{2+} entry, that trigger exocytosis. Experiments in which Ca^{2+} was suddenly removed in the presence of carbachol demonstrate that the continued presence of Ca^{2+} is required to maintain secretion (Holz and Blok, unpublished observations). Sustained Ca^{2+}-dependent phosphorylation of a protein of molecular weight 56,000–60,000 within chromaffin cells has been demonstrated to be well correlated with secretion, and may be one of the biochemical reactions involved in exocytosis (Holz et al., 1980; Amy and Kirshner, 1981). Other experiments are under way to investigate further the intracellular, Ca^{2+}-dependent reactions that underlie secretion.

After our study was completed we learned of a study on cultured bovine chromaffin cells (Kilpatrick et al., 1982) with results very similar to those in the present work.

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