# $Ca^{2+}$-dependent and -independent Release of Neurotransmitters from PC12 Cells: A Role for Protein Kinase C Activation?

T. POZZAN,* G. GATTI,‡§ N. DOZIO,‡§ L. M. VICENTINI,‡ and J. MELDOLESI‡§
*Institute of General Pathology, National Research Council Center for the Physiology of Mitochondria, University of Padova, Padova, Italy; and ‡Department of Medical Pharmacology and §National Research Council Center of Cytopharmacology, University of Milan, Milan, Italy

ABSTRACT The intracellular mechanisms regulating the process of evoked neurotransmitter release were studied in the cloned neurosecretory cell line PC12. Various agents were employed that were known, from previous studies in other systems, to stimulate release in a manner either strictly dependent or independent of the concentration of extracellular $Ca^{2+}$, $[Ca^{2+}]_o$. Three parameters were investigated in cells suspended in either $Ca^{2+}$-containing or $Ca^{2+}$-free Krebs-Ringer media: release of previously accumulated [$^3$H]dopamine; average free cytoplasmic $Ca^{2+}$ concentration, $[Ca^{2+}]_i$ (measured by the quin2 technique); and cell ultrastructure, with special reference to the number and structure of secretion granules. The release induced by the ionophores transporting monovalent cations, X537A and monensin, occurred concomitantly with profound alterations of secretory granule structure (swelling and dissolution of the dense core). These results suggest that the effect of these drugs is due primarily to leakage of dopamine from granules to the cytoplasm and extracellular space. In contrast, the changes induced by other stimulatory drugs used concerned not the structure but the number of secretory granules, indicating that with these drugs stimulation of exocytosis is the phenomenon underlying the increased transmitter release. The release response induced by the $Ca^{2+}$-ionophore ionomycin was dependent on $[Ca^{2+}]_o$, occurred rapidly, was concomitant with a marked rise of $[Ca^{2+}]_i$, and ceased after 1–2 min even though $[Ca^{2+}]_i$ remained elevated for many minutes. 12-O-tetradecanoylphorbol, 13-acetate and diacylglycerol (both of which are known as activators of protein kinase C) induced slow responses almost completely independent of $[Ca^{2+}]_o$ and not accompanied by changes of $[Ca^{2+}]_i$. Combination of an activator of protein kinase C with a low concentration of ionomycin failed to modify the $[Ca^{2+}]_i$ rise induced by the ionophore, but elicited a marked potentiation of the release response, which was two- to fourfold larger than the sum of the responses elicited separately by either drugs. Thus, activation of protein kinase C seems to play an important role in the regulation of exocytosis in neurosecretory cells, possibly by increasing and maintaining the sensitivity to $Ca^{2+}$ of the intracellular apparatus regulating granule discharge by exocytosis.

During the last two decades, considerable attention has been focused on the process of evoked transmitter release from nerve terminals and neurosecretory cells (see reference 33 for a recent review). Two important aspects of such a process now appear firmly established. The first concerns the mechanism of the release that in many (possibly all) systems has been demonstrated to occur in quanta by exocytosis, i.e., fusion of synaptic vesicles or neurosecretory granules with the plasma membrane, followed by the release of their content to the extracellular space (6, 7, 38). The second aspect concerns the key role played by $Ca^{2+}$. Depolarization, whether induced by action potential invasion of synaptic terminals or by other means, is followed rapidly by the opening of the voltage-dependent $Ca^{2+}$ channels. The consequent inward flow of



THE JOURNAL OF CELL BIOLOGY · VOLUME 99 AUGUST 1984 628–638

$Ca^{2+}$ along its steep electrochemical gradient results in a rise of the cytoplasmic free $Ca^{2+}$ concentration, $[Ca^{2+}]_i$. Although the link between the two phenomena remains undefined, the available evidence indicates that the rise of $[Ca^{2+}]_i$ can by itself trigger the exocytotic response (14, 16, 20, 24, 33). Yet, the possibility that additional mechanisms could co-exist and cooperate with the $[Ca^{2+}]_i$-dependent mechanism in the regulation of neurotransmitter release has been repeatedly considered. In fact, a variety of treatments (1, 2, 4, 11, 13, 23, 31, 36, 42) (see below for details) was found to stimulate release from various preparations even when the concentration of $Ca^{2+}$ in the extracellular fluid, $[Ca^{2+}]_o$, was lowered by $Ca^{2+}$-chelating agents. However, nerve terminals and neurosecretory cells contain large amounts of calcium bound to molecules or segregated within membrane-bounded compartments (3, 21, 25). Redistribution of this stored $Ca^{2+}$ to the cytoplasm would certainly be sufficient to raise $[Ca^{2+}]_i$. Thus, unless $[Ca^{2+}]_i$ is directly measured, the $Ca^{2+}$ independence of a release response occurring in low $[Ca^{2+}]_o$ cannot be ascertained.

Recently, two experimental tools have become available for the study of neurotransmitter release. First, cloned neurosecretory cells can be used which, while endowed with features typical of nerve cells, grow rapidly and are homogeneous. The second tool is a fluorimetric technique for measuring $[Ca^{2+}]_i$ which relies on the use of quin2 (30, 40, 41). The hydrophobic acetoxymethyl tetraester of this fluorescent $Ca^{2+}$ indicator penetrates the plasma membrane by diffusion and is then cleaved within the cytoplasm by unspecific esterases to yield the hydrophilic probe. Thus, quin2 can be loaded into cells of any size, which can then be studied in suspension by conventional fluorimetric analyses (30, 40, 41).

In a previous study (22) we used the quin2 technique in a line of rat pheochromocytoma cells (named PC12, which resemble undifferentiated sympatoblasts [8, 9, 32]) as well as in synaptosomes isolated from the guinea pig brain. Release was triggered by a presynaptic neurotoxin, $\alpha$-latrotoxin of the black widow spider venom, which stimulates exocytosis both with and without $Ca^{2+}$ in the incubation medium (13, 22, 23, 43). We found that a toxin-induced $[Ca^{2+}]_i$ rise occurred in the $Ca^{2+}$-containing, but not in the $Ca^{2+}$-free, medium (22). These results demonstrated that the release of neurotransmitter can indeed be stimulated in a genuinely $[Ca^{2+}]_i$-independent fashion. The data reported in this paper confirm and extend this conclusion. We used PC12 cells exposed to a variety of pharmacological agents that, from studies in other systems, were known to stimulate release responses either independent or strictly dependent on the presence of $Ca^{2+}$ in the incubation medium. Our data suggest the possible involvement of the recently discovered lipid-activated protein kinase, protein kinase C (27), as a possible mediator of $Ca^{2+}$-independent release responses in PC12 cells.

## MATERIALS AND METHODS

*Incubation Media:* Two media were used. Complete modified Krebs-Ringer medium (KR)[1] included (in mmol/liter) NaCl, 125; KCl 5; $MgSO_4$ and $KH_2PO_4$, 1.2; $NaHCO_3$, 5; $CaCl_2$, 1; HEPES-NaOH buffer, pH 7.4, 25; glucose, 6. The $Ca^{2+}$-free KR medium differed from complete KR by having no $Ca^{2+}$ added, 2.4 mM $MgSO_4$, and 1 mM EGTA.

*Cells:* PC12 cells were grown as monolayers in Falcon plastic flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) as described by Greene and Tischler (9). At the beginning of the experiments they were detached by gently streaming complete KR medium onto the surface of the monolayer, transferred to siliconized tubes, and recovered by centrifugation (300 *g*, 5 min).

*[$^3$H]Dopamine ([$^3$H]DA) Release:* Pelletted cells were resuspended by gentle swirling into oxygenated complete KR medium (~3 × $10^7$ cells/ml) supplemented with ascorbic acid (0.1 mg/ml), the monoamine oxidase inhibitor pargyline (0.1 mg/ml), and [$^3$H]DA (3 × $10^{-7}$ M) and incubated at 37°C for 30 min. The cell suspensions were then diluted 10-fold with KR medium, divided into four to six aliquots, centrifuged, and stored as pellets at room temperature for up to 90 min. Pellets were resuspended in 3-5 ml of oxygenated KR or $Ca^{2+}$-free KR medium, both supplemented with desmethylimipramine ($10^{-6}$ M, to inhibit amine re-uptake). They were dissociated into single (or small groups of) cells by drawing the suspensions five times in and out of a plastic syringe fitted with three autopipette tips in series. Drugs were added dissolved in either water or DMSO. Controls received the solvent only (final concentration: 1%). Viability of the cells was routinely measured by trypan blue exclusion. After dissociation, the percentage of trypan blue-positive cells in the suspension was typically 10-15%. Unless otherwise indicated this value changed only slightly during the incubation at 37°C.

In the concentration-response experiments, 200-$\mu$l incubation mixtures (containing 3.5–5.5 × $10^5$ cells and 10-20 × $10^3$ cpm) were incubated at 37°C for 10 min in disposable Eppendorf tubes, then chilled in ice and centrifuged (10,500 *g*, 30 s). The pellets were rinsed with 400 $\mu$l of cold medium, recentrifuged, resuspended in 200 $\mu$l of water and finally mixed with 4 ml of New England Nuclear Atomlight (New England Nuclear, Boston, MA). In the time-course experiments, incubations were in siliconized glass vials (cell concentration: 2.5-3.5 × $10^6$ cells/ml). 150-$\mu$l aliquots of these suspensions were withdrawn and rapidly centrifuged through a layer of mineral oil. The incubation medium remaining above the oil layer was removed, the surface of the layer was carefully rinsed, the layer was aspirated, and the cell pellet was solubilized and counted as described above. For further details see reference 23.

*Quin2 Measurement of $[Ca^{2+}]_i$:* PC12 cells, suspended in RPMI 1640 medium buffered with HEPES, pH 7.4 (1-2 × $10^7$ cells/ml), were mixed with a 0.5-1% vol of 10 mM quin2 acetoxymethylester in DMSO and incubated at 37°C for 1 h. Before use the cells were pelleted, resuspended in either KR or $Ca^{2+}$-free KR (2-3 × $10^6$ cells/ml), and dissociated (see above). Assays were carried out in a thermostated cuvette equipped with magnetic stirring with a Perkin-Elmer 650-40 spectrofluorimeter (Perkin-Elmer Corp., Eden Prairie, MN; excitation: 339 ± 2; emission: 492 ± 10 nm). Drugs dissolved in water or DMSO were added directly to the cuvette (final solvent concentration: 1%). Calibration of the fluorescent signal was made exactly as described in reference 22. Whenever necessary the data were corrected for changes in cell autofluorescence.

*Electron Microscopy:* Aliquots of cells, detached from the plastic dishes, dissociated, and incubated with or without drugs as described above were fixed for 30 min at 0°C with a mixture of glutaraldehyde (1.5%) and formaldehyde (2.5%, freshly prepared from paraformaldehyde) in 125 mM phosphate buffer, pH 7.4. Fixed cells were washed with the buffer, postfixed with 1% $OsO_4$ in phosphate buffer, block-stained with Mg uranyl acetate, and embedded in Epon. Thin sections were doubly stained with uranyl acetate and lead citrate (for details, see reference 43).

For measuring the numerical density (number per unit of cytoplasmic area) of secretion granules in sectioned cell profiles, we used the procedure described in reference 43. In brief, two blocks were sectioned for each sample, and thin sections recovered onto 100-mesh, Formvar-coated grids. One of these sections was chosen by inspection at very low magnification, and all cell profiles appearing in that section were photographed at constant magnification (× 6,500). The number of secretion granules was counted on pictures printed at the final magnification of 18,000. Clearly disrupted cells were not considered in the analysis. Cytoplasmic areas were measured with a Kontron-Zeiss MOP 01 Digiplan apparatus.

*Materials:* The following materials were obtained from the sources indicated below: 7,8-[$^3$H]DA HCl (specific radioactivity, 50 Ci/mmol): Amersham International, U.K.; pargyline, X537A, and desmethylimipramine: Ciba-Geigy (Basel, Switzerland); *p*-hydroxymercuriphenil-sulfonic acid (pHMPS), monensin, 12-*O*-tetradecanoylphorbol,13-acetate (TPA), diamide: Sigma Chemical Co., (St. Louis, MO); ouabain: Boheringer GmbH (Mannheim, Federal Republic of Germany [FRG]); erythrosine B: Aldrich Europe (Beerse, Belgium); RPMI 1640 medium: Flow Laboratories (Milan, Italy). 1-oleoyl,2-acetylglycerol (DG) and quin2 acetoxymethylester were the kind gift of Dr. R.

[1] *Abbreviations used in this paper*: DA, dopamine; DG, 1-oleoyl,2-acetylglycerol; KR, modified Krebs-Ringer medium; pHMPs, *p*-hydroxymercuriphenyl-sulfonic acid; TPA, 12-*O*-tetradecanoylphorbol,13-acetate.

Y. Tsien, University of California at Berkeley. Ionomycin was a gift of Dr. C. M. Liu, Roche Institute of Molecular Biology (Nutley, NJ). All other chemicals were reagent grade and were purchased from Merck AG (Darmstadt, FRG).

## RESULTS

### Control Cells

A detailed characterization of the PC12 cell suspension system has already been given elsewhere (23, 43). [$^3$H]DA taken up by these cells rapidly equilibrates with the endogenous pools of neurotransmitter (8, 9, 32). Release of the accumulated tracer occurs at a very low rate from unstimulated cells. Cells suspended in $Ca^{2+}$-containing medium respond rapidly to depolarizing concentrations of $K^+$ by releasing 10-15% of the accumulated [$^3$H]DA within 1-2 min whereas cells in $Ca^{2+}$-free medium do not (22, 23). If, however, the high $K^+$ stimulation is continued, the release tends to level off (8, 22). The average $[Ca^{2+}]_i$ of unstimulated PC12 cells is 109 ± 3 nM. After application of high $K^+$, this level increases rapidly three- to fourfold, reaching a plateau which then declines slowly over the following several minutes (22).

The ultrastructure of control PC12 cells has been described (32, 39, 43). In the present study, considerable attention is given to secretory granules which appear as small, spherical or ovoidal profiles, often concentrated in the cytoplasmic rim adjacent to the plasma membrane and in the Golgi complex area. Granules are composed of an electron-dense, homogeneous core, sometimes separated from the limiting membrane by a clear, narrow space. Their average numerical density (number of granules per unit area) in sectioned cytoplasmic profiles is 1.47 ± 0.12/$\mu m^2$. This value remains unchanged after incubation up to 30 min in either KR or $Ca^{2+}$-free KR (Table I). Other organelles germane to our study are the Golgi complex, composed of four to eight stacked cisternae and a large population of tubules and vesicles (some of which are coated), and mitochondria (Fig. 1*A*). PC12 cells, incubated up to 2 h in $Ca^{2+}$-free KR medium, show no major ultrastructural changes with respect to unincubated cells, except for moderate swelling of the stacked Golgi cisternae (Fig. 1*B*).

TABLE I

*Numerical Density of Secretion Granules in Sectioned Cytoplasmic Profiles of Cells Exposed to Various Treatments*

| Treatment | Incubation medium | Number of profiles | Total area analyzed | Granule density |
|---|---|---|---|---|
| | | | *nm* | *% SE* |
| —* | KR | 86 | 1,063 | 100 ± 8.0 |
| —* | Ca-free KR | 77 | 1,420 | 106 ± 6.8 |
| pHMPS | | | | |
| $10^{-3}$ M | KR | 30 | 705 | 65.7 ± 10.5 |
| 10 min | | | | |
| Erythrosine B | | | | |
| $5 \times 10^{-4}$ M | KR | 28 | 530 | 58.0 ± 13.4 |
| 30 min | | | | |
| Ionomycin | | | | |
| $1 \times 10^{-7}$ M | KR | 44 | 600 | 84.7 ± 10.8 |
| 10 min | | | | |
| $5 \times 10^{-7}$ M | KR | 32 | 604 | 43.0 ± 9.9 |
| 10 min | | | | |
| TPA | | | | |
| $3 \times 10^{-8}$ M | KR | 41 | 682 | 67.7 ± 10.3 |
| 10 min | Ca-free KR | 35 | 574 | 71.2 ± 9.5 |
| DG | | | | |
| $1.5 \times 10^{-4}$ M | KR | 48 | 525 | 97.6 ± 10.6 |
| 10 min | | | | |
| Ionomycin + TPA | | | | |
| $1 \times 10^{-7}$ M, $1 \times 10^{-8}$ M | KR | 47 | 886 | 40.6 ± 5.9 |
| 10 min | | | | |
| Ionomycin + DG | | | | |
| $1 \times 10^{-7}$ M, $1.5 \times 10^{-4}$ M | KR | 49 | 931 | 61.0 ± 8.7 |
| 10 min | | | | |

Numerical density is given in number of granules per unit area. 100% = 1.47 granules/$\mu m^2$ of cytoplasmic profile area.

* Controls included samples incubated for either 10 or 30 min. No significant difference in granule density was found between these two groups considered separately.

### Quin2-loaded Cells

Because of the use of the quin2 technique in our study, it was important to characterize the PC12 cells that were loaded with the fluorescent probe under the conditions used. Previous studies demonstrated that when the loading is performed in $Ca^{2+}$-containing media, the cell is able to compensate for the generation of the high-affinity $Ca^{2+}$ chelator by increasing the total cytoplasmic calcium, so that $[Ca^{2+}]_i$ remains unchanged (22, 30, 40, 41). We previously showed that the release response brought about in quin2-loaded PC12 cells by high $K^+$ and $\alpha$-latrotoxin was not significantly different from that in control cells (22). We have extended this work to the stimulatory drugs now used and have obtained similar results. In addition, the ultrastructure of the quin2-loaded cells has been investigated. Although the technique has already been applied to a variety of cell types, no information on its possible ultrastructural effects has yet been reported. As illustrated in Fig. 2*A*, no appreciable difference was found in PC12 cells loaded with quin2 with respect to the controls (Fig. 1*A*).

Quin2 can be used not only to measure $[Ca^{2+}]_i$ but also to control and modify this parameter. In particular, when the loading is performed in $Ca^{2+}$-free media, homeostasis is not maintained and $[Ca^{2+}]_i$ is decreased to very low levels (30). In the present work, lowering of $[Ca^{2+}]_i$ to ~5-10 nM (see Fig. 14) was obtained in PC12 cells by adding 5 mM EGTA to the RPMI 1640 medium used for loading. Henceforth, we refer to the PC12 cells loaded under these conditions as low $[Ca^{2+}]_i$ cells. Fig. 2*B* shows that the ultrastructure of low $[Ca^{2+}]_i$ cells is very similar to that of cells incubated in $Ca^{2+}$-free KR without quin2 loading, with moderate swelling of Golgi cisternae.

### Cells Treated with Stimulatory Agents

A long list of treatments was previously reported to stimulate the release of neurotransmitters from various experimental preparations (most often neuromuscular junctions of frogs, mice, and rats, and brain slices or synaptosome suspensions of rats, mice, and guinea pigs) even in $Ca^{2+}$-free incubation media (1, 2, 4, 11, 13, 31, 36, 42). Many such treatments have now been applied to PC12 cells. Incubations in either complete or $Ca^{2+}$-free KR media lasted from 1 to 30 min. Under these conditions, three treatments found to be active in other preparations (4, 11, 36) were unable to stimulate [$^3$H]DA release from PC12 cells: ouabain (0.03 to 1 mM), $LaCl_3$ (0.03 to 1 mM), and sucrose hypertonicity (100 and 300 mM) (data not shown). Moreover, the thiol-oxidizing agent diamide (42), which was able to stimulate [$^3$H]DA release both in complete and $Ca^{2+}$-free KR medium (Fig. 3*A*), induced severe toxicity

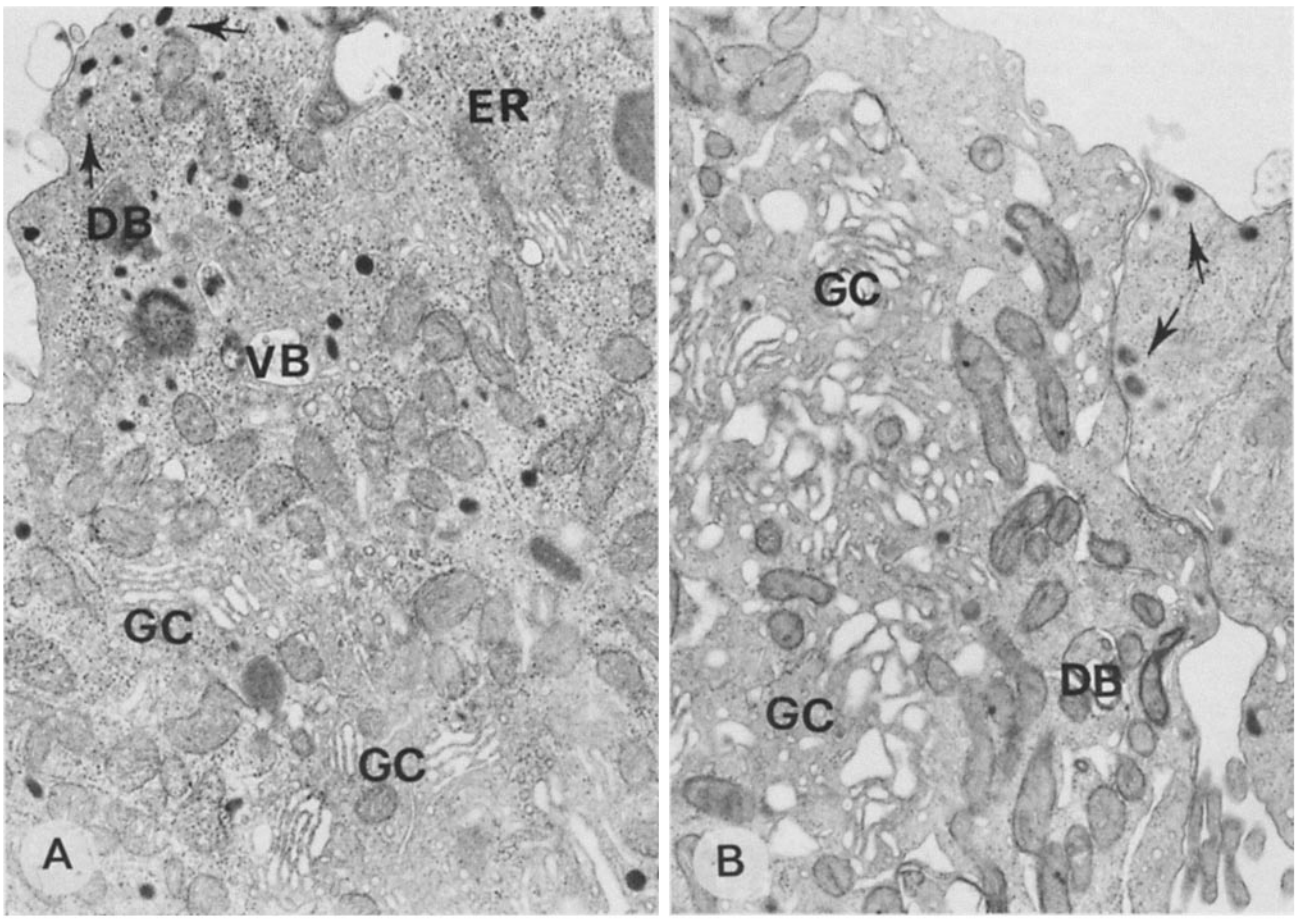


FIGURE 1 Thin-section electron microscopy of PC12 cells. *A* illustrates the Golgi area of a control cell incubated for 10 min in complete KR medium. The Golgi complex (*GC*) is composed of four to six stacked cisternae (which are dilated at their edges), tubules, and vesicles. Numerous dense granules, distributed throughout the cytoplasm, are slightly more concentrated beneath the plasma membrane (arrows). The field also includes mitochondria, endoplasmic reticulum cisternae (*ER*), and multivesicular (*VB*) and dense (*DB*) bodies. *B* shows parts of two adjacent PC12 cells incubated for 30 min in KR-EGTA. Notice the swelling of the Golgi cisternae (*GC*). × 22,500.

at all concentrations used, with marked increase of the percentage of trypan blue-positive cells and profound ultrastructural alterations of the surviving cells.

### *pHMPS and Erythrosine B*

The mechanisms by which these two drugs stimulate transmitter release independently of $[Ca^{2+}]_o$ are still undefined. pHMPS, a thiol-reducing agent, has been reported not to penetrate into living cells (2); erythrosine B, a food dye, is believed to act intracellularly (1). Concentrations of these drugs in the 0.1–1 mM range stimulated the release of [$^3$H]-DA from PC12 cells. In the $Ca^{2+}$-free medium the responses were almost identical to those in complete KR (Fig. 3, *B* and *C*). Electron microscopy of treated cells failed to reveal drastic changes of the ultrastructure, except for a marked swelling of mitochondria observed in some of the cells treated with erythrosine B (not shown). The numerical density of secretion granules was decreased in rough proportion to the release responses measured biochemically (compare Table I with Fig. 3). 0.5 mM concentration of pHMPS applied in complete KR induced a clear (~fourfold), verapamil-inhibitable increase of $[Ca^{2+}]_i$ (Fig. 4*A*). In contrast, pHMPS in $Ca^{2+}$-free medium had no effect on $[Ca^{2+}]_i$ (not shown). The effects of erythrosine B on $[Ca^{2+}]_i$ could not be established owing to interference of this intensely red drug with the quin2 fluorescence.

### *X537A and Monensin*

These two cation ionophores have different specificity. X537A exchanges $H^+$ for various monovalent and divalent cations, whereas monensin exchanges $H^+$ only with monovalent cations, with marked preference for $Na^+$ (12, 28, 29, 37). In rat brain synaptosomes, the release of catecholamines induced by X537A has been attributed to the ionophore effect of the drug for amines (i.e., redistribution of the transmitter from granules to the cytoplasm and the extracellular space), rather than to stimulation of exocytosis (12). The mechanism of the monensin-induced stimulation of catecholamine release from neurosecretory cells (whether transmitter redistribution [29] or stimulation of exocytosis [37]) is still debated.

The results that we obtained in PC12 cells with X537A and monensin were similar. Marked release of [$^3$H]DA was observed which, at low concentrations, was appreciably smaller in the complete than in $Ca^{2+}$-free KR media (Fig. 5). Time-course experiments revealed that the effect brought about by optimal concentrations of the drugs begun shortly after their

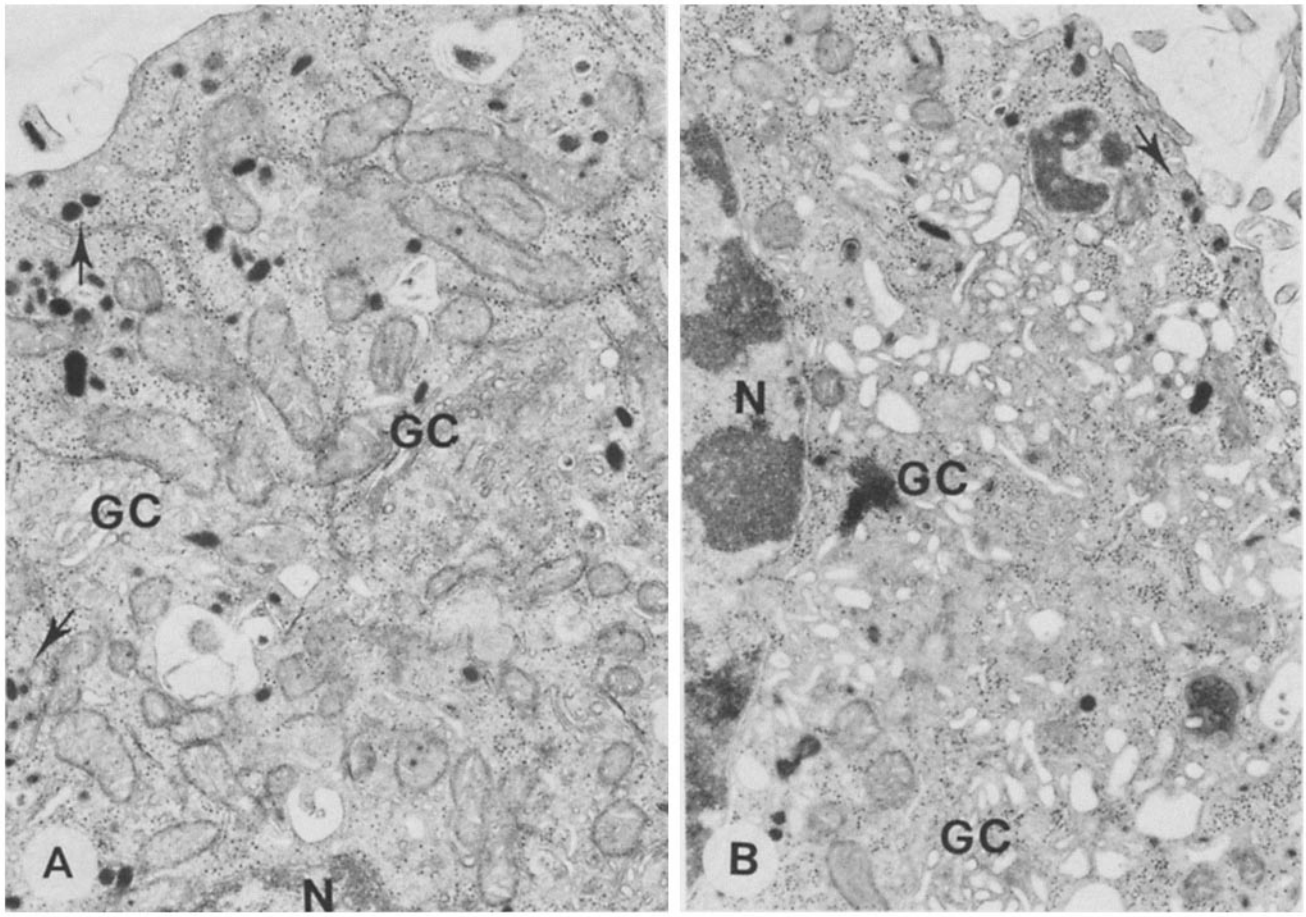


FIGURE 2 Thin-section electron microscopy of PC12 cells loaded with quin2. *A* shows part of the cytoplasm of a cell loaded in a $Ca^{2+}$-containing medium. Notice the numerous electron-dense secretory granules (more concentrated near the cell surface, arrows), the stacked Golgi cisternae (*GC*), and mitochondria. *B* shows a low $[Ca^{2+}]_i$ cell, i.e., a cell loaded with quin2 in a $Ca^{2+}$-free medium. The general structure of the cell appears well preserved, but Golgi cisternae (*GC*) are swollen. *N*, nucleus. Other labels are as in Fig. 1*A*. × 23,000.

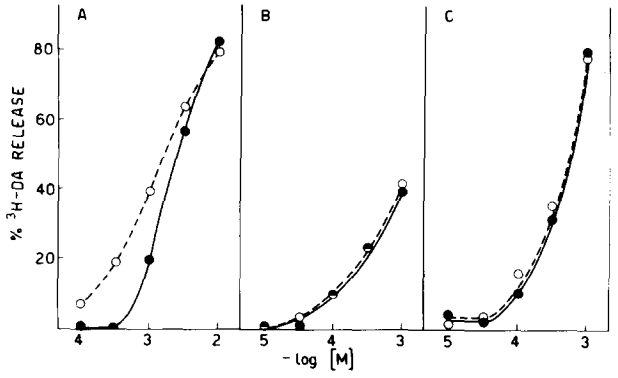


FIGURE 3 Concentration dependence of [$^3$H]DA release induced in PC12 cells by diamide (*A*), pHMPS (*B*), and erythrosine B (*C*). Cell suspensions in either complete (●) or $Ca^{2+}$-free (○) KR medium were incubated at 37°C for 10 (*B*) or 30 (*A* and *C*) min. Values shown are averages of three to four experiments from which the release occurring in unstimulated cells has been subtracted.

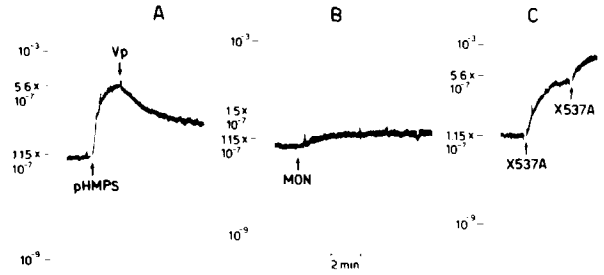


FIGURE 4 $[Ca^{2+}]_i$ changes in PC12 cells exposed to (*A*) pHMPS, $0.5 \times 10^3$ M followed by verapamil ($V_p$) $2 \times 10^{-5}$ M; (*B*) monensin (*Mon*), $1 \times 10^6$ M; (*C*) X537A, $1 \times 10^6$ M, two additions. Values shown to the left of each trace represent the $[Ca^{2+}]_i$ (M) calibration scale.

application and continued at high rates until most of the stored [$^3$H]DA was discharged (not shown). With both drugs, sustained increases of $[Ca^{2+}]_i$ were observed when the cells were incubated in complete KR (Fig. 4, *B* and *C*). However, the effect of X537A was much larger than that of monensin. In $Ca^{2+}$-free KR, X537A induced smaller and transient $[Ca^{2+}]_i$ rises, similar to other more specific $Ca^{2+}$ ionophores (see Fig. 9*D* for ionomycin). The effect of monensin in $Ca^{2+}$-free KR was very small but still appreciable (not shown). Already at 5 min after the application of either X537A or monensin, most secretory granules appeared swollen, sometimes with widening of the clear halo around the core. The core showed a markedly reduced density; in many granules it appeared

partially dissolved (Fig. 6*A*). At 30 min only few swollen granules could be recognized. Moreover, Golgi cisternae were no longer visible. Both structures were replaced by a population of apparently empty vacuoles, measuring up to 2 μm diam, which were distributed throughout the whole cytoplasm (Fig. 6*B*). Nuclei, mitochondria, and endoplasmic reticulum cisternae remained normal. With either drug, no morphological difference was seen between cells treated in the complete or in the $Ca^{2+}$-free KR medium.

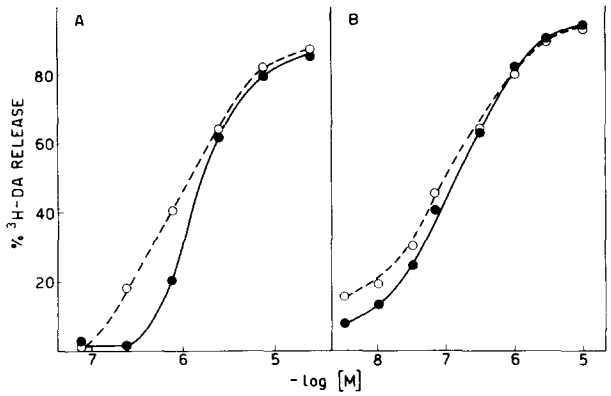


FIGURE 5 Concentration dependence of [$^3$H]DA release induced in PC12 cells by X537A (*A*) and monensin (*B*). Cell suspensions in either complete (●) or Ca-free (○) KR medium were incubated at 37°C for 10 min. Values shown are averages of three (*A*) or six (*B*) experiments from which basal release has been subtracted.

## *Ionomycin, TPA, and DG*

The mechanism of action of these agents is quite different. Ionomycin is a $Ca^{2+}$ ionophore. Because of its hydrophobicity it inserts rapidly into cellular membranes and promotes an electron-neutral $Ca^{2+}$-$H^+$ exchange. Affinity of ionomycin for divalent cations other than $Ca^{2+}$ is low (28, 34). TPA is a hydrophobic molecule which, when applied to cells, induces a pleiotropic response (activation of ion fluxes, enzyme activities, and cell growth). Recent evidence indicates that the direct target of TPA is protein kinase C, where TPA acts as the stable analog of the physiological lipid metabolite activator diacylglycerol (5, 15, 26, 27, 34, 35).

The effects of ionomycin were greatly influenced by the presence of $Ca^{2+}$ in the incubation fluid (Figs. 7–10). In complete KR, stimulation of [$^3$H]DA release was appreciable already at 0.1 μM ionomycin (~10% in 10 min), and was greatly increased at higher concentrations (Fig. 7). The re-

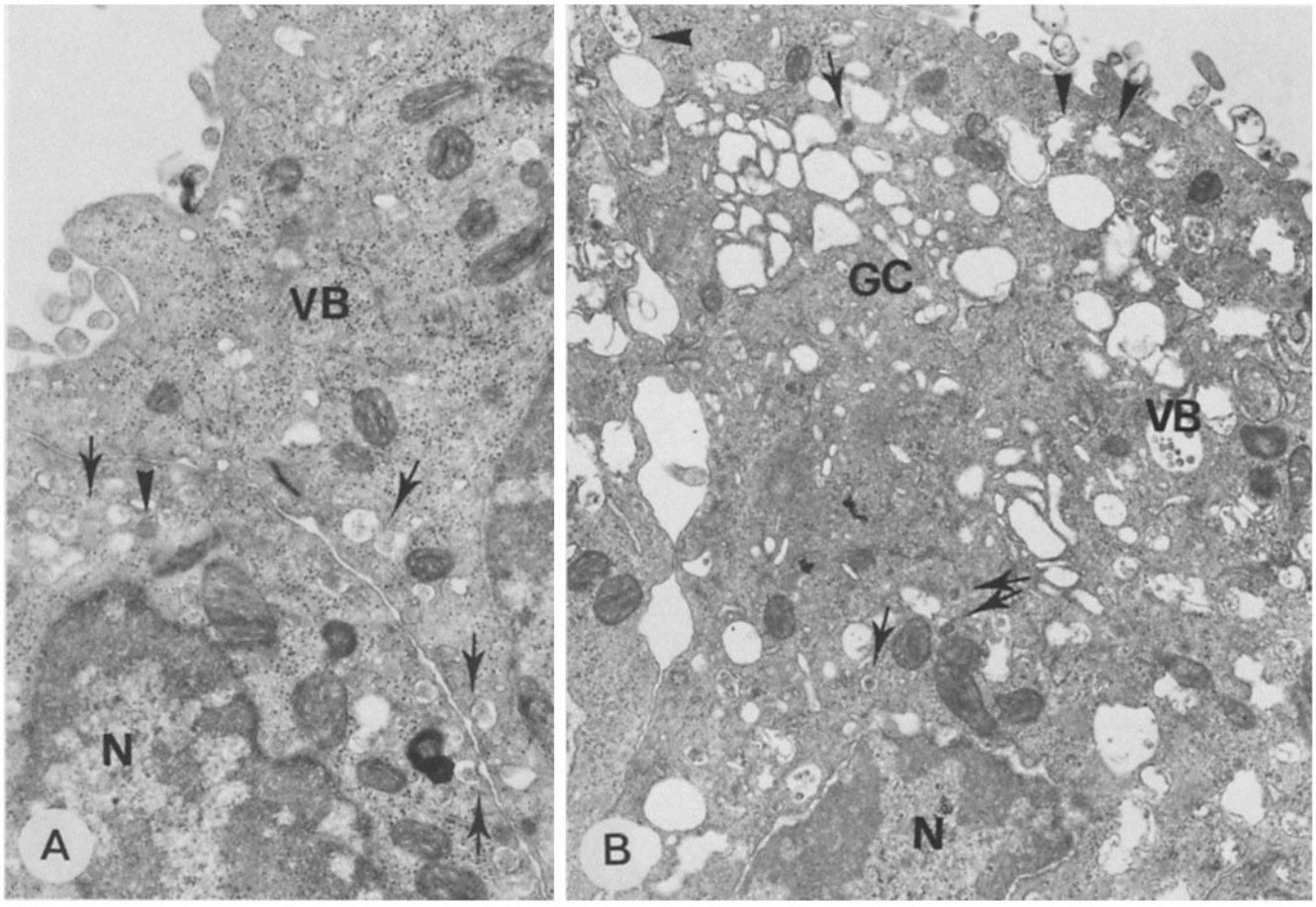


FIGURE 6 Effects of monensin ($3 \times 10^{-7}$ M) on PC12 cell ultrastructure. In *A* two cells treated with the drug for 5 min exhibit marked changes of the structure of secretion granules: swelling, widening of the clear space around the granule core, decreased density, and partial dissolution of the latter (arrows). Only a few granules appear better preserved (arrowheads). × 21,500. The cells shown in *B* were treated with monensin for 30 min. Notice the disordered organization of the Golgi complex (GC), where the stacked cisternae appear swollen or replaced by vacuoles. Granules similar to those illustrated in *A* are still visible (arrows); others have probably turned into partially or completely empty vacuoles (arrowheads). × 19,000. (Other labels as in Figs. 1 and 2).

sponse to ionomycin was rapid. Most of the drug-induced release had already occurred after 1-2 min (Fig. 8). However, a $Ca^{2+}$-free KR, a relatively small release of [$^3$H]DA was observed and only at a concentration of ≥0.5 μM (Fig. 7). Fig. 9 illustrates the effects of ionomycin on $[Ca^{2+}]_i$. In complete KR the drug induced prompt, concentration-dependent rises which, at concentrations >0.2 μM, were maintained by the cells for several minutes. In $Ca^{2+}$-free KR medium the $[Ca^{2+}]_i$ also increased. However, the rise was smaller and transient (back to the unstimulated level within 0.5-1 min [Fig. 9]).

When applied in complete KR at concentrations of 0.1-0.3 μM, ionomycin had no effect on the general organization of PC12 cells (Fig. 10*A*). No change of the secretion granule structure was visible, while their numerical density (Table I) was decreased in parallel with the [$^3$H]DA release effect (Fig. 7). At higher concentrations of ionomycin, however, signs of cytotoxicity became evident. At 0.5 μM and, more markedly, at 1 μM, the number of trypan blue-positive cells, measured at the end of the incubations (10 min), was increased over the controls, although moderately (~10–30%). A considerable proportion of the remaining cells showed clumping of chromatin in nuclei, detachment of ribosomes from endoplasmic reticulum cisternae, extensive vacuolization of the cytoplasm, and formation of thick cytoplasmic projections at the cell surface. In contrast, no major alterations of mitochondria were seen (not shown). The morphology of individual secretory granules was unchanged, while their average number per unit of cytoplasmic area was decreased (Table I). Cells exposed to 1 μM ionomycin in $Ca^{2+}$-free KR maintained their granules and showed no obvious signs of cytotoxicity.

Both TPA and DG were able to stimulate the release of [$^3$H]DA from PC12 cells. As can be seen in Fig. 11, the effect of TPA was appreciable at 10 nM (10-15% release in 10 min) and became considerable at ≥100 nM. Incubation in $Ca^{2+}$-free KR reduced the release response to TPA only slightly.

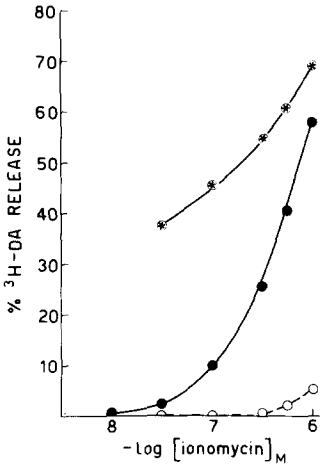


FIGURE 7 Concentration dependence of [$^3$H]DA release induced in PC12 cells by ionomycin ± TPA. The solid and open circles show the results obtained with cells suspended in either complete or $Ca^{2+}$-free medium, respectively, and incubated with ionomycin at 37°C for 10 min. In the experiments illustrated by the enclosed asterisks, the cells, suspended in complete KR, were treated for the same time with ionomycin (at the concentrations specified on the abscissa) together with TPA, $10^{-8}$ M. The effects of TPA alone are illustrated in Figs. 11 and 12. Values shown are averages of three to six experiments subtracted of basal release.

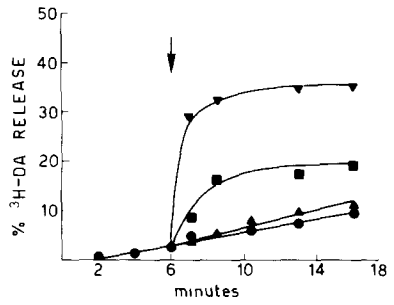


FIGURE 8 Time course of [$^3$H]DA release from PC12 cells treated with ionomycin. Cells suspended in complete KR were incubated at 37°C. Addition of ionomycin (▼, 3 × $10^{-7}$ M; ■, $10^{-7}$ M; ▲, 3 × $10^{-8}$ M) is marked by the arrow; ●, controls. The values shown are averages of two to three experiments.

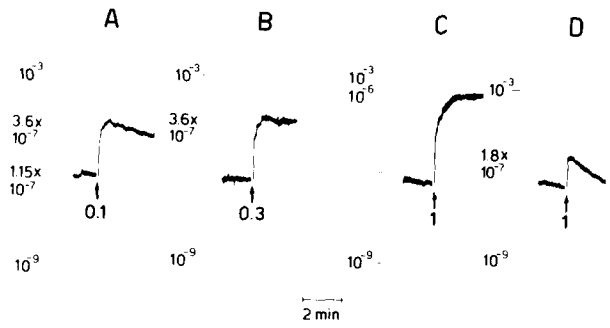


FIGURE 9 $[Ca^{2+}]_i$ changes in PC12 cells exposed to ionomycin in complete or $Ca^{2+}$-free KR. 2 × $10^6$ cells/ml containing ~0.5 nmol of quin2/$10^6$ cells were treated with ionomycin at the concentrations specified below the arrows (μM). *A–C*, complete KR; *D*, $Ca^{2+}$-free KR. $[Ca^{2+}]_i$ calibration is indicated as in Fig. 4.

DG, on the other hand, had a small, but highly reproducible effect at concentrations >10 μM, with and without $Ca^{2+}$ in the medium (5-7% release in 10 min). The time course of the responses to 10 nM TPA and 150 μM DG was different from that observed with ionomycin. As shown in Fig. 12, the rates of release elicited by either agent were higher with respect to the controls not only during the initial minutes of their activity but also during the entire time period investigated (10 min).

A further series of experiments was carried out to investigate the dependence of the TPA and DG stimulation of transmitter release on $[Ca^{2+}]_i$. For this purpose $[Ca^{2+}]_i$ was either increased by ionomycin or decreased by loading the cells with quin2 in $Ca^{2+}$-free medium. The effects of TPA and DG in cells with high $[Ca^{2+}]_i$ are illustrated in Figs. 7, 12, and 13. Combination of ionomycin in the 30-300 nM range with either 10 nm TPA or 150 μM DG in complete KR medium elicited release responses distinctly (two- to threefold) larger than the sum of the effects produced separately by the individual agents (compare the data in Figs. 7 and 12). An even larger (almost fourfold) synergism was observed when very low concentrations of ionomycin and TPA were used (30 and 3 nM, respectively) (data not shown). At high ionomycin concentrations (≥0.5 μM), the synergism decreased and simple additivity was finally observed (Fig. 7).

The combination of TPA plus 0.1 μM ionomycin was applied to PC12 cells also in $Ca^{2+}$-free medium. With 10 nM TPA no synergistic effect was detected, i.e., the release was the same as with TPA alone, since 0.1 μM ionomycin is without effect in the $Ca^{2+}$-free medium (Fig. 7). However,

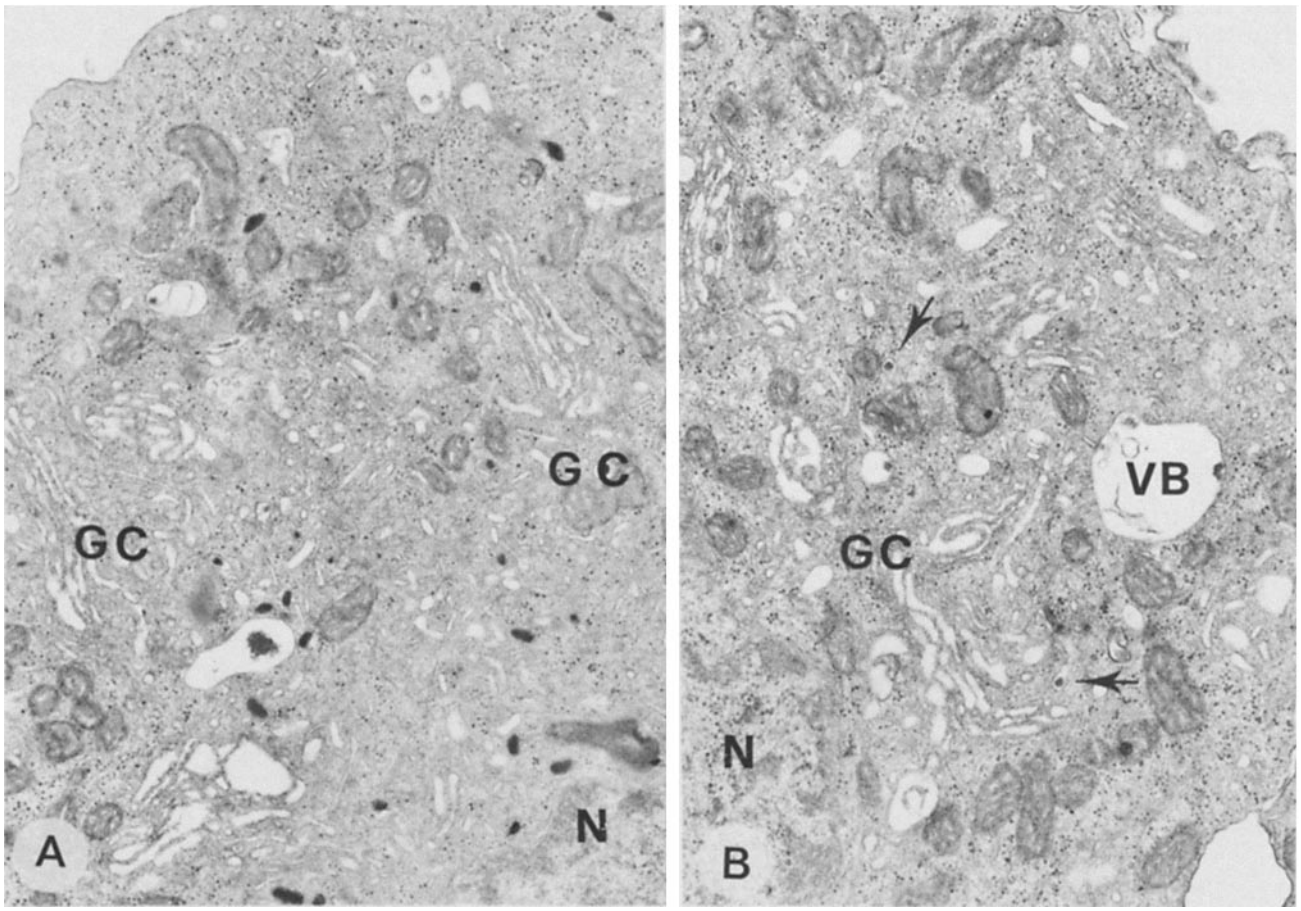


FIGURE 10 Effects of ionomycin and TPA plus ionomycin on PC12 cell ultrastructure. *A* shows that cells treated for 10 min with ionomycin ($10^{-7}$ M) maintain a normal organization. The number of their secretion granules is slightly decreased compared with controls, especially in the region beneath the plasmalemma. × 21,500. After the combined treatment with ionomycin ($10^{-7}$ M) and TPA ($10^{-8}$ M) (*B*), the decrease of secretion granules is marked. The few granules shown in the figure are located in the Golgi area (arrows). × 22,500.

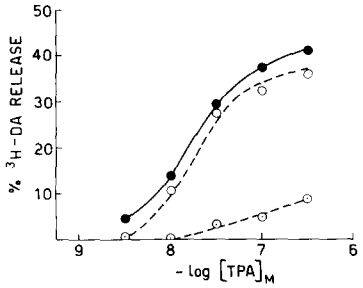


FIGURE 11 Concentration dependence of [$^3$H]DA release induced in PC12 cells by TPA. Cell suspensions in either complete (●) or $Ca^{2+}$-free (○) KR were incubated at 37°C for 10 min. ⊙ shows the results obtained with low $[Ca^{2+}]_i$ cells suspended in $Ca^{2+}$-free medium. For convenience these cells were loaded concomitantly with both [$^3$H]DA and quin2 by incubation at 37°C for 1 h in RPM 1640 medium + 5 mM EGTA supplemented with the two tracers, pargyline and ascorbic acid, at the concentrations specified in Materials and Methods. Values shown are averages of two experiments subtracted of basal release.

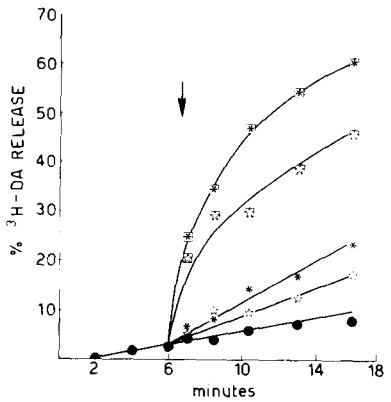


FIGURE 12 Time course of [$^3$H]DA release induced in PC12 cells by treatment with TPA or DG, or combinations of these drugs with ionomycin. Cells suspended in KR were incubated at 37°C. After 6 min (arrow) TPA ($10^{-8}$ M, asterisk), DG ($1.5 \times 10^{-4}$ M, star), or either one together with ionomycin ($10^{-7}$ M, enclosed asterisk and enclosed star, respectively) were added. The solid circles indicate controls. Values shown are averages of three experiments.

when the concentration of TPA was raised to 0.1 μM, the combination with 0.1 μM ionomycin induced a release effect distinctly larger (+40%) than that by TPA alone (not shown).

When TPA was applied in the $Ca^{2+}$-free medium to low $[Ca^{2+}]_i$ cells, the [$^3$H]DA release effect of the drug was markedly reduced, but a small stimulation appeared at TPA concentrations in the 0.1 μM range (Figs. 11 and 13). Such an inhibition of the TPA effect was not due to unspecific damage of low $[Ca^{2+}]_i$ cells because treatment with ionomycin plus

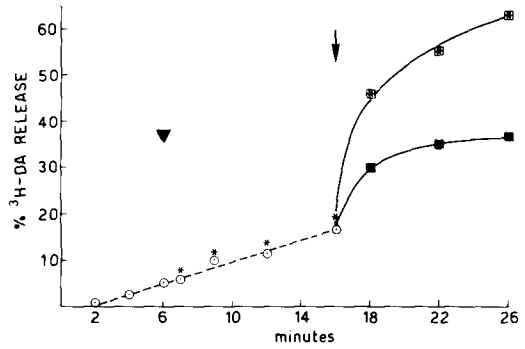


FIGURE 13 Time course of [$^3$H]DA release from low $[Ca^{2+}]_i$ PC12 cells treated with TPA and ionomycin. Duplicate aliquots of low $[Ca^{2+}]_i$ cells suspended in $Ca^{2+}$-free KR were incubated at 37°C. After 6 min (arrowhead), TPA ($10^{-7}$ M) was added to one aliquot (asterisk), while the other (enclosed dot) received the solvent alone. After 16 min of incubation (arrow), ionomycin ($10^{-7}$ M) was added to both TPA-treated (enclosed asterisk) and control (square) aliquots together with $CaCl_2$ (3 mM). Values shown are averages of two experiments.

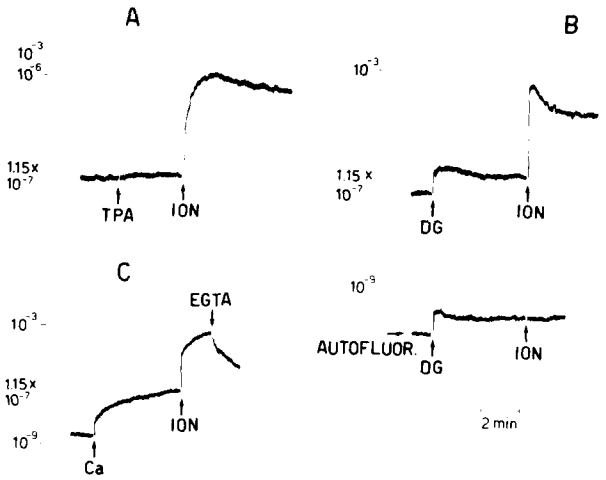


FIGURE 14 $[Ca^{2+}]_i$ changes in PC12 cells treated with TPA, DG, and ionomycin. (*A*) $2 \times 10^6$ cells/ml containing 0.25 nmol of quin2/$10^6$ cells in complete KR medium were treated with TPA, $10^{-7}$ M, followed by ionomycin (*ION*), $3 \times 10^{-7}$ M. (*B*) DG, $1.5 \times 10^4$ M, followed by ionomycin (*ION*), $1.5 \times 10^7$ M, was applied to parallel aliquots of quin2 loaded cells (same concentrations as in *A*, *upper trace*) and unloaded cells (*lower trace*). Note that the apparent rise of $[Ca^{2+}]_i$ induced by DG is entirely accounted for by an increase of autofluorescence. (*C*) $1 \times 10^6$/ml low $[Ca^{2+}]_i$ PC12 cells (i.e., cells loaded with quin2 acetoxymethylester in a $Ca^{2+}$-free medium) in $Ca^{2+}$-free KR were treated first with $CaCl_2$ (Ca, 2 mM), then with ionomycin (*ION*, $5 \times 10^{-7}$ M), and finally with EGTA, $5 \times 10^{-3}$ M. In separate experiments TPA ($3 \times 10^{-8}$ M) did not modify the $[Ca^{2+}]_i$ of low $[Ca^{2+}]_i$ cells (not shown). Calibration is indicated as in Fig. 4.

$Ca^{2+}$ induced vigorous [$^3$H]DA release responses, which were greatly potentiated in the cells pretreated with TPA (Fig. 13).

TPA and DG, at the concentrations used for the release experiments, failed to modify the resting $[Ca^{2+}]_i$ of PC12 cells, or to affect the rise induced by ionomycin (Fig. 14, *A* and *B*). Fig. 14*C* shows that low $[Ca^{2+}]_i$ cells have an average $[Ca^{2+}]_i$ <10 nM. Upon addition of $Ca^{2+}$ to the medium, this value increased rapidly towards the resting level, and was further greatly elevated by the subsequent addition of ionomycin.

Neither TPA nor DG, in the experimental conditions used, induced changes of the cell ultrastructure (not shown), except for the decrease of the granule numerical density induced by TPA in parallel with its effect on [$^3$H]DA release (Table I). Even cells exposed to the combination of 0.1 μM ionomycin plus either 10 nM TPA (Fig. 10*B*) or 150 μM DG appeared well preserved. The synergism noticed for [$^3$H]DA release also appeared for the granule numerical density, which was greatly reduced (by ~60 and 40%, respectively; Table I).

## DISCUSSION

In the present work, suspensions of PC12 cells were exposed to a variety of treatments previously shown in other systems to stimulate the release of neurotransmitters. Many of these treatments stimulated the release of [$^3$H]DA from PC12 cells. However, the mechanism of these responses was not the same in all cases. Ionophores transporting monovalent cations, X537A and monensin, induced marked alterations of secretory granule structure, with progressive disappearance of the dense core and swelling of the organelle to yield large, apparently empty vacuoles. This result was expected with X537A which is known from studies in other systems to transport amines across the granule and plasma membrane (12, 28). Previous data on monensin (which has a high affinity for $Na^+$) were conflicting. Results of transmitter release in isolated chromaffin cells were interpreted as stimulation of exocytosis (37). But in PC12 cells, redistribution and leakage of catecholamines was reported to be responsible for the monensin-induced DA release (29). Our present morphological results support the latter interpretation.

As for the $[Ca^{2+}]_i$ rises induced by the two drugs, their mechanism and significance appear to be different. X537A, which is also a $Ca^{2+}$ ionophore, induced rises qualitatively similar to those of ionomycin. By analogy with the results obtained with ionomycin, we believe that at relatively high concentrations (>0.5 μM), X537A also stimulates exocytosis (see also reference 28). In contrast, the small increase of $[Ca^{2+}]_i$ brought about by monensin appears to be too low to be directly responsible for stimulating exocytosis. Redistribution of $Ca^{2+}$ from intracellular stores and inhibition of $Na^+/Ca^{2+}$ exchange across the plasma membrane could contribute to the $[Ca^{2+}]_i$ rise induced by these two drugs.

A second group of agents tested decreased the numerical density of secretory granules without changing their structure. The observation that the decrease of granule density approximately correlated with the amount of [$^3$H]DA release strongly suggests that the effects of these agents occurred through stimulation of exocytosis. For one such agent, erythrosine B, the information we obtained in PC12 cells does not go beyond the demonstration of its ability to stimulate transmitter release independently of $[Ca^{2+}]_i$, as has been previously shown by others in a different system (1).

As far as the other agents are concerned, the data on transmitter release should be considered in conjunction with those on $[Ca^{2+}]_i$. Ionomycin, which stimulates release in an almost exclusively $Ca^{2+}$-dependent fashion, was found to induce large, persistent $[Ca^{2+}]_i$ rises when applied in complete KR, and considerable, although transient, rises in $Ca^{2+}$-free KR. pHMPS, on the other hand, was found to increase $[Ca^{2+}]_i$ when applied in complete KR, and have no such effect in $Ca^{2+}$-free medium, although it was able to stimulate [$^3$H]DA release equally well in either medium. Finally, TPA and DG induced release responses almost completely independent of $[Ca^{2+}]_o$, and without modifying the $[Ca^{2+}]_i$ level of cells.

In order to fully understand the meaning of these results, it is necessary to know in detail the mechanisms of action and intracellular targets of the various drugs used. pHMPS is a thiol-reducing agent which is believed to act extracellularly by causing the reduction of one or more disulfide bonds in proteins exposed at the outer surface of the plasma membrane. In addition, pHMPS was found to depolarize synaptosomes (2). Our data are consistent with the latter finding because the verapamil-inhibitable rise of $[Ca^{2+}]_i$ in complete KR is a typical effect of depolarizing agents in PC12 cells (22). However, the $[Ca^{2+}]_i$ increase appears to play no major role in the pHMPS-induced release response, because that response was not appreciably different in cells incubated with or without $Ca^{2+}$ in the medium. At the moment we have no explanation to account for such an intriguing result.

The mechanism of action of the other drugs used is better known. All the effects of ionomycin, a specific $Ca^{2+}$ ionophore, are believed to be mediated by its changing $[Ca^{2+}]_i$ levels (28, 34); the effects of TPA and DG are believed to be mediated by the activation of the recently discovered protein kinase C (5, 15, 19, 26, 27, 34, 35). As the activation of the latter enzyme is a very complex process, the mechanisms of action of ionomycin on the one hand and TPA and DG on the other cannot be considered entirely separate. Purified protein kinase C requires acidic phospholipids and $Ca^{2+}$ in micromolar concentrations for maximal activity (27). Inside the cell, resting $[Ca^{2+}]_i$ would be too low for activation to occur. However, the affinity of the enzyme for $Ca^{2+}$ is increased considerably upon binding of the activators of another class, which includes TPA and diacylglycerols (5, 15, 19, 27, 34, 35). Receptor binding of a variety of hormones and neurotransmitters activates the hydrolysis of phosphoinositides, with consequent release of diacylglycerols within the cell. In addition, most of these hormones and neurotransmitters are believed to raise $[Ca^{2+}]$ within cells as well. Thus, raised $[Ca^{2+}]_i$ and diacylglycerols can lead to physiological activation of protein kinase C (15, 27, 34, 35). In our experimental conditions, both ionomycin (at concentrations sufficient to induce large increases of $[Ca^{2+}]_i$) and TPA and DG were expected to cause activation of the enzyme especially when applied in combination.

The ability of TPA to stimulate secretion by exocytosis has been known for several years (10). However, the key role played by protein kinase C in this process has been appreciated only recently. In human platelets, release responses of similar magnitude were induced by either ionomycin or TPA and DG (34, 35), with and without elevation of $[Ca^{2+}]_i$, respectively (34). In addition, Rink et al. (34) reported synergistic responses elicited by ionomycin administered after TPA or DG treatment, with no concomitant change of the $[Ca^{2+}]_i$ elevation induced by the ionophore. The present work extends this approach to a line of neurosecretory cells that has features in common with neurons, i.e., a cell type in which fluctuations of $[Ca^{2+}]_i$ are believed to be the main regulatory mechanism of exocytosis (8, 9, 32). The results we have obtained in PC12 cells concur with the previous data on platelets (34, 35). In addition, an important novel aspect was revealed by our studies. We observed that the release responses elicited by ionomycin on the one hand, TPA and DG on the other, had distinctly different features. In particular, those induced by ionomycin were rapid, but tended to level off after 1-2 min, although $[Ca^{2+}]_i$ remained elevated. These results are similar to those obtained previously in PC12 cells exposed to depolarizing concentrations of $K^+$ (22). Thus, the trigger effect of high $[Ca^{2+}]_i$ in exocytosis appears to inactivate within a very short time (a few minutes at the most) (see also references 18 and 24). TPA and DG induced responses that were similar in rate (both were slow and sustained), but were markedly different quantitatively. The high potency and efficacy of TPA was expected because this drug binds with high affinity to protein kinase C within the cell and is metabolized only slowly (5, 19, 27). The diacylglycerol we used (1-oleoyl,2-acetylglycerol) was chosen because of its ability to stimulate release when administered exogenously at considerable concentration (34, 35).

The synergistic responses evoked by the concomitant application of ionomycin with TPA or DG also revealed interesting features. As already observed in platelets (34), the synergism was more marked at low ionomycin concentrations, and tended to disappear at high concentrations. The synergism, evident already during the first 1-2 min, when release was markedly stimulated even by ionomycin alone, became very large during the next few minutes, at times when the responses to ionomycin alone tended to level off. Therefore, the stimulatory effects of the combined drug treatments continued at high rates. These responses resemble closely, both in rate and extent, those elicited in PC12 cells by optimal concentrations of the black widow spider toxin $\alpha$-latrotoxin, when applied in complete KR medium (22, 23). It is interesting that under those conditions the toxin causes a large $[Ca^{2+}]_i$ rise and in addition activates a $[Ca^{2+}]_i$-independent mechanism, which accounts for the persistence of a considerable part of its action even in $Ca^{2+}$-free media (22, 23, 43).

Taken together, the data that we have reported indicate that two types of transmitter release responses can be evoked by pharmacological treatments in PC12 cells, and that synergism results from the concomitant stimulation of these two types of responses. Because of their distinct differences, it is unlikely that the two types of responses are mediated intracellularly by one single mechanism. Thus, two mechanisms seem to co-exist. One could be mediated directly by $[Ca^{2+}]_i$, the other operating through the activation of protein kinase C. A question that remains open is whether or not either one of these two mechanisms can operate in an entirely independent fashion. As already mentioned, high $[Ca^{2+}]_i$ itself can activate protein kinase C. On the other hand, evidence has been reported in chromaffin cells made leaky by high voltage discharge suggesting that activation of the enzyme can increase the potency of $[Ca^{2+}]_i$ in exocytosis (17). Investigating this problem is difficult because no specific inhibitors of protein kinase C are available. Moreover, in intact PC12 cells it has yet not been possible to measure the activity of the enzyme. The results we obtained by the use of low $[Ca^{2+}]_i$ cells (in which the $[Ca^{2+}]_i$ mechanism is expected to be inactive) were inconclusive. In these cells the effect of TPA was greatly decreased, but a small response could still be elicited.

In conclusion, the study of [$^3$H]DA release and $[Ca^{2+}]_i$ in PC12 cells exposed to various pharmacological treatments in $Ca^{2+}$-containing and $Ca^{2+}$-free media has provided evidence implicating protein kinase C in the regulation of transmitter release by exocytosis. This interpretation is entirely based on a pharmacological criterion, i.e., it rests on the specificity of the enzyme activators used, TPA and DG. If protein kinase C indeed plays the role suggested by our data, it might mediate not only the response elicited by the two activators but also

those by other agents (discussed above) that cause $[Ca^{2+}]_i$-independent release of neurotransmitters. Preliminary data indicate that in PC12 cells the strong, specific stimulator $\alpha$-latrotoxin (22, 23) triggers the turnover of phosphoinositides (the process responsible for the physiological activation of protein kinase C), whereas ionomycin does not.

The work in PC12 cells was initiated to generate ideas that we are now planning to test in neurons. At the present time, a direct extrapolation to synapses of the data obtained in the cloned cells would be unjustified. However, the possibility that protein kinase C plays a role in presynaptic physiology appears reasonable because high concentrations of the enzyme are contained in the brain (26, 27) with enrichment in synaptosomes, where a specific substrate has been identified (44). Whether protein kinase C is involved in the regulation of transmitter release and/or other functions in synapses remains a task for future work.

The technical collaboration of Mr. G. Ronconi, P. Tinelli, and F. Crippa is gratefully acknowledged. We thank N. Borgese and H. Scheer for critically reading the text, I. Ruffoni for typing it, and P. Calissano for introducing PC12 cells to our laboratory.

This work was supported in part by grants from the Italian Ministry of Education, Membrane Biology and Pathology Program, and Programma Finalizzato Oncologia of the Italian National Research Council (to T. Pozzan and J. Meldolesi).

*Received for publication 23 November 1983, and in revised form 21 March 1984.*

## REFERENCES

1. Augustine, G. J., and H. Levitan. 1983. Presynaptic effects of erythrosine B at the frog neuromuscular junction. Ion and photon sensitivity. *J. Physiol. (Lond.).* 334:65–77.
2. Baba, A., J. S. Fischerman, and J. R. Cooper. 1979. Action of sulfydryl reagents on cholinergic mechanisms in synaptosomes. *Biochem. Pharmacol.* 28:1879–1883.
3. Blaustein, M. P., R. W. Ratzlaff, and S. E. Schweitzer. 1978. Calcium buffering in presynaptic nerve terminals: evidence for involvement of a non-mitochondrial ATP-dependent sequestration mechanism. *J. Gen. Physiol.* 72:15–41.
4. Birks, R. I., and M. W. Cohen. 1968. The influence of internal sodium on the behaviour of motor nerve endings. *Proc. R. Soc. Lond. B Biol. Sci.* 170:401–421.
5. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.
6. Ceccarelli, B., and W. P. Hurlbut. 1980. Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* 60:396–441.
7. Douglas, W. W. 1974. Involvement of calcium in exocytosis and in the exocytosis-vesiculation sequence. *Biochem. Soc. Symp.* 39:1–28.
8. Greene, L. A., and G. Rein. 1977. Release, storage and uptake of catecholamines by a clonal line of nerve growth factor (NGF) responsive pheochromocytoma cells. *Brain Res.* 129:247–263.
9. Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2474–2478.
10. Gunther, G. R. 1981. Effect of 12-O-tetradecanoyl-phorbol-13-acetate on $Ca^{2+}$ efflux and protein discharge in pancreatic acini. *J. Biol. Chem.* 256:12040–12045.
11. Heuser, J. E., and R. Miledi. 1971. Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc. Lond. B Biol. Sci.* 179:247–260.
12. Holtz, R. W. 1975. The release of dopamine from synaptosomes from rat striatum by the ionophores X537A and A23187. *Biochim. Biophys. Acta.* 375:138–152.
13. Hurlbut, W. P., and B. Ceccarelli. 1979. Use of black widow spider venom to study the release of neurotransmitters. *In* Neurotoxins: Tools in Neurobiology. B. Ceccarelli and F. Clementi, editors. Raven Press, New York. 87–115.
14. Katz, G., and R. Miledi. 1970. Spontaneous and evoked activity of motor nerve endings in calcium Ringer. *J. Physiol. (Lond.).* 203:689–706.
15. Kishimoto, A., Y. Takai, T. Mori, W. Kikkawa, and Y. Nishizuka. 1980. Activation of calcium and phospholipid dependent protein kinase by diacyl-glycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* 255:2273–2276.
16. Knight, D. E., and P. F. Baker. 1982. Calcium dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric field. *J. Membr. Biol.* 68:107–140.
17. Knight, D. E., and P. F. Baker. 1983. The phorbol ester TPA increases the affinity of exocytosis for calcium in "leaky" adrenal medullary cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 160:98–100.
18. Knight, D. E., and N. T. Kesteven. 1983. Evoked transient intracellular free $Ca^{2+}$ changes and secretion in isolated bovine adrenal medullary cells. *Proc. R. Soc. Lond. B Biol. Sci.* 218:177–199.
19. Leach, K. L., M. L. James, and P. M. Blumberg. 1983. Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. Natl. Acad. Sci. USA.* 80:4208–4212.
20. Llinas, R. 1979. The role of calcium in neural function. *In* The Neurosciences. Fourth Study Program. F. O. Schmitt and F. G. Worden, editors. Massachusetts Institute of Technology Press, Boston. 555–571.
21. McGraw, C. F., A. V. Somlyo, and M. P. Blaustein. 1980. Localization of calcium in presynaptic nerve terminals: an ultrastructural and electron microprobe analysis. *J. Cell Biol.* 85:228–241.
22. Meldolesi, J., W. B. Huttner, R. Y. Tsien, and T. Pozzan. 1984. Free cytoplasmic $Ca^{2+}$ and neurotransmitter release. Studies on PC12 cells and synaptosomes exposed to $\alpha$latrotoxin. *Proc. Natl. Acad. Sci. USA.* 81:620–624.
23. Meldolesi, J., L. Madeddu, M. Torda, G. Gatti, and E. Niutta. 1983. The effect of $\alpha$latrotoxin on the neurosecretory PC12 cell line: studies on toxin binding and stimulation of transmitter release. *Neuroscience.* 10:997–1009.
24. Miledi, R., and I. Parker. 1981. Calcium transients recorded with arsenazo III in the presynaptic terminal of the squid giant synapse. *Proc. R. Soc. Lond. B Biol. Sci.* 212:197–211.
25. Nicholls, D. G., and K. E. O. Akerman. 1981. Biochemical approaches to the study of cytosolic calcium regulation in nerve endings. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 296:115–122.
26. Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36–40.
27. Nishizuka, Y. 1983. Phospholipid degradation and signal translation for protein phosphorylation. *Trends Biochem. Sci.* 8:13–16.
28. Perlman, R. L., A. F. Cossi, and L. W. Role. 1980. Mechanisms of ionophore-induced catecholamine secretion. *J. Pharmacol. Exp. Ther.* 213:241–246.
29. Perlman, R. L., B. E. Sheard, A. S. Tischler, and P. W. L. Kwan. 1982. Monensin depletes PC12 pheochromocytoma cells of catecholamines and of chromaffin granules. *Neurosci. Lett.* 29:177–182.
30. Pozzan, T., P. Arslan, R. Y. Tsien, and T. J. Rink. 1982. Anti-immunoglobulin, cytoplasmic free calcium, and capping in B lymphocytes. *J. Cell Biol.* 94:335–340.
31. Quastel, D. M. J., J. T. Hackett, and J. D. Cook. 1971. Calcium: is it required for transmitter release? *Science (Wash. DC).* 172:1034–1036.
32. Rebois, R. V., E. E. Reynolds, L. Toll, and B. D. Howard. 1980. Storage of dopamine and acetylcholine in granules of PC12, a clonal pheochromocytoma cell line. *Biochemistry.* 19:1240–1248.
33. Reichardt, L. F., and R. B. Kelly. 1983. A molecular description of nerve terminal function. *Annu. Rev. Biochem.* 52:871–926.
34. Rink, T. J., A. Sanchez, and T. J. Hallam. 1983. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature (Lond.).* 305:317–319.
35. Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka. 1983. A role of calcium-activated, phospholipid-dependent protein kinase in human platelet activation. Comparison of thrombin and collagen actions. *J. Biol. Chem.* 258:2010–2013.
36. Shimoni, Y., E. Alnaes, and R. Rahaminoff. 1977. Is hyperosmotic neurosecretion from motor nerve endings a calcium dependent process? *Nature (Lond.).* 267:170–172.
37. Suchard, S. J., F. A. Lattanzio, R. W. Rubin, and B. C. Pressman. 1982. Stimulation of catecholamine secretion from cultured chromaffin cells by an ionophore-mediated rise in intracellular sodium. *J. Cell Biol.* 94:531–539.
38. Thureson-Klein, A. 1983. Exocytosis from large and small dense cored vesicles in noradrenergic nerve terminals. *Neuroscience.* 10:245–252.
39. Tischler, A. S., and L. A. Greene. 1978. Morphological and cytochemical properties of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Lab. Invest.* 39:77–89.
40. Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. T cell mitogens cause early changes in cytoplasmic free $Ca^{2+}$ and membrane potential in lymphocytes. *Nature (Lond.).* 292:68–70.
41. Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* 94:325–334.
42. Wade, P. D., L. C. Fritz, and P. Siekevitz. 1981. The effect of diamide on transmitter release and on synaptic vesicle population at vertebrate synapses. *Brain Res.* 225:357–372.
43. Watanabe, O., M. Torda, and J. Meldolesi. 1983. The effect of $\alpha$latrotoxin on the neurosecretory PC12 cell line. Electron microscopy and cytotoxicity studies. *Neuroscience.* 10:1011–1024.
44. Wu, W. C., S. I. Walaas, A. C. Nairn, and P. Greengard. 1982. Calcium/phospholipid regulates phosphorylation of a $M_r$ 87K substrate protein in brain synaptosomes. *Proc. Natl. Acad. Sci. USA.* 79:5249–5253.