

## Calcium Signaling and Exocytosis in Adrenal Chromaffin Cells

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**García, Antonio G., Antonio M. García-de-Diego, Luis Gandía, Ricardo Borges, and Javier García-Sancho.** Calcium Signaling and Exocytosis in Adrenal Chromaffin Cells. *Physiol Rev* 86: 1093–1131, 2006; doi:10.1152/physrev.00039.2005.—At a given cytosolic domain of a chromaffin cell, the rate and amplitude of the  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) depends on at least four efficient regulatory systems: 1) plasmalemmal calcium channels, 2) endoplasmic reticulum, 3) mitochondria, and 4) chromaffin vesicles. Different mammalian species express different levels of the L, N, P/Q, and R subtypes of high-voltage-activated calcium channels; in bovine and humans, P/Q channels predominate, whereas in felines and murine species, L-type channels predominate. The calcium channels in chromaffin cells are regulated by G proteins coupled to purinergic and opiate receptors, as well as by voltage and the local changes of  $[\text{Ca}^{2+}]_c$ . Chromaffin cells have been particularly useful in studying calcium channel current autoregulation by materials coreleased with catecholamines, such as ATP and opiates. Depending on the preparation (cultured cells, adrenal slices) and the stimulation pattern (action potentials, depolarizing pulses, high  $\text{K}^+$ , acetylcholine), the role of each calcium channel in controlling catecholamine release can change drastically. Targeted aequorin and confocal microscopy shows that  $\text{Ca}^{2+}$  entry through calcium channels can refill the endoplasmic reticulum (ER) to nearly millimolar concentrations, and causes the release of  $\text{Ca}^{2+}$  (CICR). Depending on its degree of filling, the ER may act as a sink or source of  $\text{Ca}^{2+}$  that modulates catecholamine release. Targeted aequorins with different  $\text{Ca}^{2+}$  affinities show that mitochondria undergo surprisingly rapid millimolar  $\text{Ca}^{2+}$  transients, upon stimulation of chromaffin cells with ACh, high  $\text{K}^+$ , or caffeine. Physiological stimuli generate  $[\text{Ca}^{2+}]_c$  microdomains in which the local subplasmalemmal  $[\text{Ca}^{2+}]_c$  rises abruptly from 0.1 to  $\sim 50 \mu\text{M}$ , triggering CICR, mitochondrial  $\text{Ca}^{2+}$  uptake, and exocytosis at nearby secretory active sites. The fact that protonophores abolish mitochondrial  $\text{Ca}^{2+}$  uptake, and increase catecholamine release three- to fivefold, support the earlier observation. This increase is probably due to acceleration of vesicle transport from a reserve pool to a ready-release vesicle pool; this transport might be controlled by  $\text{Ca}^{2+}$  redistribution to the cytoskeleton, through CICR, and/or mitochondrial  $\text{Ca}^{2+}$  release. We propose that chromaffin cells have developed functional triads that are formed by calcium channels, the ER, and the mitochondria and locally control the  $[\text{Ca}^{2+}]_c$  that regulate the early and late steps of exocytosis.

## I. INTRODUCTION

Fear, stress, or conflicts trigger a surge of the catecholamines epinephrine and norepinephrine that mobilize the body for the “fight or flight” response; the heart rate, the strength of myocardial contraction, and blood pressure increase; the blood flow switches to skeletal muscle; glucose is mobilized from the liver and rises in the circulation; and the pupils and bronchioles dilate (200). The body is thus prepared to survive by combating an enemy or to flee from danger. This highly coordinated and precise response is regulated by the sympathetic nervous system and the adrenal gland in an attempt to maintain the equilibrium of the internal milieu (48, 71).

The “fight or flight” response is the end result of a secretory event that takes place in the adrenal medulla, the inner part of the two adrenal glands located just above the kidneys. The adrenal medulla is composed of chromaffin cells that secrete epinephrine and norepinephrine. These cells are of interest not only as the basis of the “fight or flight” response, but also because they have been excellent models to study the working of other secretory cells, in particular neurons.

Feldberg et al. (141) established that the main physiological neurotransmitter at the splanchnic nerve-chromaffin cell synapse was acetylcholine. Acetylcholine causes the release of catecholamines from the gland. The secretory response is suppressed in the absence of extracellular  $\text{Ca}^{2+}$  (126), and secretion is accompanied by an enhancement of  $^{45}\text{Ca}^{2+}$  entry into chromaffin cells (125).

On the basis of these observations and other experiments, William W. Douglas (123) coined the expression *stimulus-secretion coupling* as the source of neurotransmitter and hormone secretion;  $\text{Ca}^{2+}$  was the coupling agent between the stimulus and the exocytotic response.

Because of their unlimited availability, particularly from bovine species, their common origin with sympathetic neurons in the neural crest (154), and their ease of isolation and preparation in primary cultures (238), chromaffin cells have been widely used in biochemical, electrophysiological, and neuropharmacological studies. Their usefulness has been further enhanced by the development of techniques to separate norepinephrine- from epinephrine-containing cells (274, 275). Thus fundamental findings on catecholamine synthesis, storage, and release were extrapolated, with success, from these cells to basic neurotransmission mechanisms in the central and peripheral nervous systems.

From fertilization of cells at their origin to cell death by apoptosis,  $\text{Ca}^{2+}$  are essential to multiple physiological and pathological processes (78, 309). The physiological function of chromaffin cells consists in the exocytotic release of the catecholamines epinephrine and norepinephrine into the circulation in response to stress (71). Because this release is a  $\text{Ca}^{2+}$ -dependent process (126), it is not surprising that chromaffin cells have been widely used as models to study the correlation between  $\text{Ca}^{2+}$  and exocytosis (286). They contain all the elements required for a strict control, both spatial and kinetic, of the  $\text{Ca}^{2+}$

transients required during the various steps of exocytosis (see Ref. 65 for a review).

Chromaffin cells are excitable cells and fire action potentials that open plasma membrane  $\text{Ca}^{2+}$  channels and produce  $\text{Ca}^{2+}$  entry; the resulting cytosolic  $\text{Ca}^{2+}$  signal triggers exocytosis (36, 90, 239, 308). Because cytoplasmic organelles can take up and release  $\text{Ca}^{2+}$  to the cytosol, understanding the cytosolic  $\text{Ca}^{2+}$  signal requires understanding  $\text{Ca}^{2+}$  redistribution between the cytosol and the different organelles. Being able to code one of the photoprotein aequorin genes (344, 345) has made it possible to introduce targeting sequences, and measure selective  $[\text{Ca}^{2+}]$  changes in different organelles (62, 329, 331). The practical efficiency of measuring aequorin expression has been very much improved and simplified by the use of viral vectors (14, 354), to the point where it is possible to image a single cell (387). This methodology has been recently applied to gain insight into the role of organelles in shaping  $\text{Ca}^{2+}$  signaling and exocytosis in these cells. This review focuses on the pathways for  $\text{Ca}^{2+}$  influx into the chromaffin cell, on the intracellular organelles that contribute to the redistribution of the  $\text{Ca}^{2+}$  entering the cell, and on the mechanisms that terminate the  $\text{Ca}^{2+}$  signals and extrude the cation outside the cell. Finally, by obtaining a more unified picture of  $\text{Ca}^{2+}$  signaling and exocytosis in chromaffin cells, we try to correlate these  $\text{Ca}^{2+}$  signals with the exocytotic responses.

## II. CALCIUM ENTRY THROUGH VOLTAGE-ACTIVATED CHROMAFFIN CELL CALCIUM CHANNELS

Four approaches helped with the discovery of the rich diversity of voltage-activated calcium channels in excitable cells (167, 299). The improvement of patch-clamp techniques has made it possible to characterize the biophysical properties of calcium channels (kinetics of activation, inactivation, and deactivation, voltage range for activation, conductance), both at the single-channel and at the whole cell levels (185). The invention of suitable fluorescent  $\text{Ca}^{2+}$  probes (327, 378) has allowed us to follow changes on cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) in living cells. Tracers other than  $\text{Ca}^{2+}$ , particularly  $\text{Mn}^{2+}$  (184), pass through  $\text{Ca}^{2+}$  channels (151, 384, 386) and can be extremely useful in tracing activity, since their appearance in the cytosol can be accurately followed with fluorescent probes without interference from  $\text{Ca}^{2+}$  released from the intracellular  $\text{Ca}^{2+}$  stores (17, 172). On the other hand, the isolation, purification, and synthesis of different neurotoxins have provided ligands with remarkable discrimination for different subtypes of high-threshold calcium channels (299). And finally, molecular biology and genetic approaches clarified the molecular structure of calcium channels (42).

Calcium channels are formed by a multiple subunit protein complex consisting of a pore-forming  $\alpha_1$ -subunit with several other auxiliary proteins, which include the intracellular  $\beta$ -subunit and a disulfide-linked  $\alpha\delta$ -subunit. In some tissues, a fifth subunit may exist, such as the transmembrane  $\gamma$ -subunit, which is a part of the channel complex in skeletal muscle or the neuronal p95 subunit. The functional diversity between different subtypes of calcium channels can be explained by 1) the existence of multiple genes encoding different classes of  $\alpha_1$ - and  $\beta$ -subunits as well as diverse variants from a single gene generated by alternative splicing, and 2) multiple possible combinations among the subunits which make up the channel complex.

Calcium channels can be classified according to their range of activation into two main groups: one has a low activation threshold (LVA channels; T channels) and the other has a high threshold for activation (HVA channels; L, N, P/Q, and R channels). Table 1 summarizes the properties of these channels and also presents their old and new nomenclatures.

### A. T-Type Channels

In addition to their low activation threshold, LVA calcium channels are characterized by a similar permeability to  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  (80, 152, 153). A single subtype of LVA calcium channel, termed T (for "transient" or "tiny"), has been identified. T channel's main characteristics include fast inactivation, which generates a transient current, and inactivation at holding potentials between  $-60$  and  $-50$  mV. The single conductance is around 8 pS. Pharmacologically, T-type channels can be distinguished from other subtypes because they are more sensitive to blockade by the inorganic calcium channel blocker  $\text{Ni}^{2+}$  than by  $\text{Cd}^{2+}$  (152, 153). It has also been reported that T-type channels can be blocked by *l*-octanol, amiloride, and the antihypertensive drug mibefradil (265).

T-type currents are difficult to record in chromaffin cells. Although we have detected T-type channel mRNA in bovine chromaffin cells (169), we have been unable to record T-type currents. However, there are three studies reporting T-type  $\text{Ca}^{2+}$  currents in bovine (118) and rat chromaffin cells (59, 201). It has been suggested that T-type calcium channels are mainly expressed in immature developing chromaffin cells (59). Recently, T-type channels of the  $\alpha_{1H}$  class have been found to be expressed in rat chromaffin cells exposed to cAMP (290), and those channels were found to trigger a secretory response (173). This  $\alpha_{1H}$  T-type calcium channel has also been identified in rat adrenal glomerulosa zone (340).

TABLE 1. *Calcium channel subtypes according to their  $\alpha_1$ -containing subunit*

Calcium Channel Type					
Novel nomenclature	Traditional nomenclature	Type of Current	Blockers	Activators	Tissue Location
Cav 1.1	$\alpha_{1S}$	L	Nifedipine Nisoldipine Nitrendipine	BAY K 8644 FPL64176	Skeletal muscle
Cav 1.2	$\alpha_{1C}$	L	Nifedipine Nisoldipine Nitrendipine	BAY K 8644 FPL64176 PCA50941	Heart Smooth muscle Brain Pituitary Adrenal medulla
Cav 1.3	$\alpha_{1D}$	L	Nifedipine Calcicludine	BAY K 8644 FPL64176 PCA50941	Brain Pancreas Adrenal medulla Cochlea Kidney Ovary Retina
Cav 1.4	$\alpha_{1F}$	L	Nifedipine	BAY K 8644 FPL64176	
Cav 2.1	$\alpha_{1A}$	P/Q	$\omega$ -Aga-IVA $\omega$ -Ctx-MVIIC $\omega$ -Ctx-MVIID		Cerebellum Pituitary Cochlea Adrenal medulla
Cav 2.2	$\alpha_{1B}$	N	$\omega$ -Ctx-GVIA $\omega$ -Ctx-MVIA		Brain Peripheral nervous system Adrenal medulla
Cav 2.3	$\alpha_{1E}$	R	SNX-482		Brain Cochlea Retina Heart Pituitary Adrenal medulla
Cav 3.1	$\alpha_{1G}$	T	Mibefradil		Brain Peripheral nervous system Adrenal medulla
Cav 3.2	$\alpha_{1H}$	T			Heart Brain Kidney Liver Adrenal glomerulosa Adrenal medulla
Cav 3.3	$\alpha_{1I}$	T			Brain

$\omega$ -Aga-IVA,  $\omega$ -agatoxin IVA;  $\omega$ -Ctx-GVIA,  $\omega$ -conotoxin GVIA;  $\omega$ -Ctx-MVIA,  $\omega$ -conotoxin MVIA;  $\omega$ -Ctx-MVIIC,  $\omega$ -conotoxin MVIIC;  $\omega$ -Ctx-MVIID,  $\omega$ -conotoxin MVIID.

## B. L-Type Channels

L-type (for “long lasting”) calcium channels are kinetically characterized by showing little inactivation during depolarizing steps ( $\tau_{\text{inact}} > 500$  ms) and their lower sensitivity to depolarized holding potentials; however, when  $\text{Ca}^{2+}$  is used as a charge carrier, these channels are completely inactivated in chromaffin cells (193). Single-channel conductance is between 18 and 25 pS in 100 mM  $\text{Ba}^{2+}$ . These L-type calcium channel subtypes seem to be present in almost all excitable cells. They are the main pathway for  $\text{Ca}^{2+}$  entry in heart and smooth muscle, as well as in the control of hormone and transmitter release from endocrine cells and some neurons. Four different  $\alpha_1$ -subunits ( $\alpha_{1C}$ ,  $\alpha_{1D}$ ,  $\alpha_{1F}$ , and  $\alpha_{1S}$ ) are responsible for L-type currents in different tissues (Table 1).

Pharmacologically, L-type calcium channels are characterized by their sensitivity to 1,4-dihydropyridines (DHP), whether agonists (i.e., BAY K 8644) or antagonists (i.e., nifedipine, nitrendipine, nisoldipine, nimodipine, flunarizine; Table 1). DHP agonists prolong the mean time duration of channel opening (291), typically observed in whole cell electrophysiological recordings as a prolongation of tail currents (315). Other organic compounds that effectively block L-type calcium channels (149, 355) include the arylalkylamines (i.e., verapamil) and benzothiazepines (i.e., diltiazem). They are particularly useful in cardiac and smooth muscle cells, where they exert negative inotropic and relaxing effects. Some piperazine derivatives (cinnarizine, flunarizine, dotarizine, R56865) also block L-type calcium channels, but they also block other subtypes of calcium channels and thus have been consid-

ered “wide-spectrum” calcium channel blockers (164, 234, 389). The same is true for imidazole antimycotics (386). Some toxins have also been shown to block L-type calcium channels, either in a selective (calciseptine and calcicludine) or a nonselective manner ( $\omega$ -agatoxin IA,  $\omega$ -agatoxin IIA, and  $\omega$ -agatoxin IIIA).

L-type currents have been characterized in bovine (9, 30, 31, 57, 58, 157), rat (158, 322), mouse (191), pig (226), cat (5), and human chromaffin cells (162, 322). Recent studies have presented molecular evidence that L-type currents in chromaffin cells are carried out by two different calcium channels:  $\alpha_{1C}$  and  $\alpha_{1D}$  (42).

### C. N-Type Channels

N-type calcium channels inactivate faster than L-type channels and do not persist at less negative holding potentials. In some preparations N-type calcium channels can contain a noninactivating component, for instance, in bovine chromaffin cells, in which N-type channels have been described as “nonclassical N-type” (31). Pharmacologically, N-type calcium channels are characterized by irreversible blockade by the *Conus geographus* toxin  $\omega$ -conotoxin GVIA (219, 291, 299) and reversible blockade by the *Conus magus* toxin  $\omega$ -conotoxin MVIIA (Table 1) (383, 385). Other wide-spectrum toxins such as  $\omega$ -conotoxin MVIIC and  $\omega$ -conotoxin MVIID (267, 385) can also block N-type calcium channels in a nonselective manner. This is also the case for  $\omega$ -agatoxin IIA,  $\omega$ -agatoxin IIIA, and  $\omega$ -grammotoxin SIA (isolated from the venom of the tarantula *Grammostola spatulata*).

N-channel currents have been characterized in chromaffin cells of various species including bovine (31, 249), pig (226), cat (5), rat (158), mouse (191), and human (162). This current suffers voltage-dependent inactivation (152, 390, but see Ref. 31) and is irreversibly blocked by  $\omega$ -conotoxin GVIA (298) and  $\omega$ -conotoxin MVIIC (160, 198) or reversibly blocked by  $\omega$ -conotoxin MVIID (160, 267).

### D. P-Type Channels

P-type calcium channels were first described by Llinás et al. (242) in cerebellar Purkinje cells, in which  $\text{Ca}^{2+}$  currents were resistant to blockade by DHP and  $\omega$ -conotoxin GVIA. The toxin fraction from the venom of the funnel web spider *Agelenopsis aperta* (FTX) effectively blocked this current. These results led to the suggestion of the existence of a new subtype of HVA calcium channel, which was named P (for “Purkinje”). P-type calcium channels are characterized by their relative insensitivity to changes in the holding potential and because they do not inactivate during depolarizing steps (262–264, 324). Pharmacologically, P-type calcium channels can be

blocked by FTX, its synthetic analog sFTX, and by  $\omega$ -agatoxin IVA at nanomolar concentrations (Table 1). P-type calcium channels can also be blocked in a nonselective manner by  $\omega$ -conotoxin MVIIC (198, 267),  $\omega$ -conotoxin MVIID, and by  $\omega$ -grammotoxin SVIA (231, 312, 313, 380).

Nanomolar concentrations of  $\omega$ -agatoxin IVA, known to fully and selectively block P-type channels (262, 264), cause only a 5–10% blockade of calcium channel current in bovine chromaffin cells (10). Previous studies reported larger contributions of P-type channels to the whole chromaffin cell  $\text{Ca}^{2+}$  currents; however, this blockade is now attributable to inhibition of Q-type channels (407) by sFTX (157) or large concentrations of  $\omega$ -agatoxin IVA (9, 26). In cat chromaffin cells, combined  $\omega$ -conotoxin GVIA plus nisoldipine blocked 90% of the current, leaving little room for P-type channels (5). In rat (158) and mouse chromaffin cells (191), the  $\omega$ -agatoxin IVA-sensitive current fraction was only 10–15%. Thus, in all species studied, P-type channels are barely expressed in chromaffin cells. This, together with the difficulty of separating the  $\alpha_{1A}$ -subunit into P- and Q-type channels (339), suggests the convenience of speaking of P/Q-type channels rather than of two separate calcium channel subtypes.

### E. Q-Type Channels

The isolation, purification, and synthesis of the toxin from the marine snail *Conus magus*  $\omega$ -conotoxin MVIIC (198, 267) led to the identification and characterization of a new subtype of HVA channel termed Q (323, 407). The characterization of Q-type calcium channels rests mainly on pharmacological criteria. Q-type channels are resistant to blockade by DHPs,  $\omega$ -conotoxin GVIA, and low concentrations (<100 nM) of  $\omega$ -agatoxin IVA, but are sensitive to  $\omega$ -conotoxin MVIIC (1–3  $\mu\text{M}$ ). Higher concentrations of  $\omega$ -agatoxin IVA (up to 2  $\mu\text{M}$ ) can also block Q-type calcium channels (407). It should be noted that these toxins are not selective for this subtype of channel since they also nonselectively block N and P channels. Other toxins that can block Q channels as well include the *Conus magus* snail toxin  $\omega$ -conotoxin MVIID (267) and the *Grammostola spatulata* tarantula toxin,  $\omega$ -grammotoxin SIA (231, 312, 313, 380).

The P/Q component of the whole cell calcium channel current has been widely studied in chromaffin cells. This component is voltage inactivated (390), and it is pharmacologically isolated by 2  $\mu\text{M}$   $\omega$ -conotoxin MVIIC,  $\omega$ -conotoxin MVIID, or  $\omega$ -agatoxin IVA. In bovine chromaffin cells,  $\omega$ -conotoxin MVIID reversibly blocks the N current, but blockade by  $\omega$ -conotoxin MVIIC is irreversible (160). Thus the use of  $\omega$ -conotoxin MVIID followed by its washout can be a convenient tool to isolate the P/Q channel. The blocking effects of  $\omega$ -conotoxin MVIIC are extraordinarily slowed down and decreased in the pres-

ence of high concentrations (i.e., more than 2 mM) of  $Ba^{2+}$  (10, 258) or  $Ca^{2+}$  (385).

## F. R-Type Channels

In neuronal tissues, a residual  $Ca^{2+}$  current, characterized by its insensitivity to blockade by DHPs,  $\omega$ -conotoxin GVIA,  $\omega$ -agatoxin IVA, and  $\omega$ -conotoxin MVIIC, has also been described and named "R type" for "resistant" (323). This subtype of calcium channel belongs to the HVA group, inactivates rapidly, and is more sensitive to blockade by  $Ni^{2+}$  than by  $Cd^{2+}$ . Newcomb et al. (289) described the first selective R channel blocker SNX-482, a peptide from the African tarantula *Hysterocrates gigas*. We found, however, that this toxin also blocks P/Q channels in the bovine chromaffin cell (23). Thus caution should be exerted when using this toxin to target R-type currents.

Differences have been reported in various laboratories concerning the expression of R-type calcium channels in chromaffin cells, and they may be due to the configuration of the patch-clamp technique (whole cell vs. perforated-patch recordings). In some initial studies, an R-type component of the calcium channel current could not be detected in bovine (9, 10, 26, 28, 30, 157, 251, 382), cat (5), human (162), pig (226), or mouse chromaffin cells (11, 191). In contrast, using the perforated-patch configuration instead of whole cell patch configuration, an R-type component was found in slices of mouse adrenal medulla and mouse chromaffin cells (11, 12). The most obvious explanation for this finding is that some soluble cytosolic factor, which is necessary for chromaffin cell R-channel activity, is dialyzed with the whole cell but not with the perforated-patch configuration. R-type currents have also been reported in rat chromaffin cells (75, 85, 201).

## G. Differences Between Species

Notable species differences have been found among the subtypes of calcium channels to be expressed by different cell types. For instance, the  $K^+$ -evoked  $Ca^{2+}$  entry in brain cortex synaptosomes is controlled by N channels in chicks and by P channels in rats (60). On the other hand, neurotransmitter release at the muscle endplate is controlled by N channels in fish (3, 140, 346) and amphibians (214) and by P channels in mammals (402).

Detailed comparative electrophysiological studies from six different mammalian species have been performed in adrenal medullary chromaffin cells (Fig. 1). L-type calcium channels account for nearly half of the whole cell calcium channel current in the cat (5), rat (158), and mouse chromaffin cells (191). But in pigs (226),

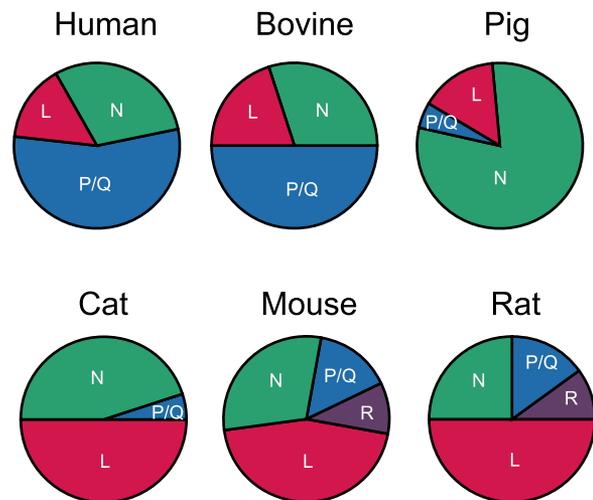


FIG. 1. Relative proportions of different neuronal calcium channel subtypes in primary cultures of chromaffin cells isolated from bovine, rat, mouse, cat, pig, and human adrenal medullary tissues. In fresh mouse adrenal slices, the R-type calcium channels carry as much as 22% of the whole cell current; no data are available in other animal species.

bovine (9, 157), and humans (162), L channels carry only 15–20% of the whole cell  $Ca^{2+}$  current.

The N channel also shows a high interspecies variability. In the pig it carries as much as 80% of the whole cell calcium channel current (226) and 45% in the cat (5); in bovine (249), rat (158), mouse (191) and human chromaffin cells (162) the N-type fraction accounts for 30% of the whole cell calcium channel current.

The fraction of current carried by P/Q channels in bovine chromaffin cells amounts to 50% (10). This fraction is even higher (60%) in human chromaffin cells (162). The opposite occurs in pig (226) and cat chromaffin cells (5) where P/Q channels carry only 5% of the current. Finally, in rat chromaffin cells, P/Q channels contribute 20% to the current (158) and ~30% in mouse chromaffin cells in the earlier study (191), but more recent studies report that P/Q channels make only 15% of the current in the mouse cells (12).

We do not yet know the physiological relevance of these extreme interspecies differences. But, surely they have clear consequences for the fine control of the differential exocytotic release of epinephrine and norepinephrine in response to different stressors. Differing autocrine/paracrine regulation by catecholamines and other coexocytotic vesicular components of the L- and non-L-types of calcium channels might be a reason. Other regulatory mechanisms (i.e., voltage- or  $Ca^{2+}$ -dependent inactivation of calcium channels) (261, 390), could also explain the preferential expression of one or another channel type in a given species. On the other hand, the selective segregation of a particular channel type to exocytotic microdomains, and the uneven geographic distribution of other channel types, might also result in a

neurosecretory cell preferentially expressing one or another type of channel. An alternative explanation for this channel diversity rests on the assumption that chromaffin cells probably have the same function in the six animal species studied to date, i.e., the sudden release of catecholamines in response to stressors. This might explain the lack of an evolutionary pressure to conserve a particular pattern of expression of calcium channel subtypes. In any case, the differences of channel type expression provide different models of chromaffin cells to study the dominant role of a calcium channel subtype in controlling exocytosis. Research in the next few years will probably develop interesting techniques with isolated adrenal slices from various species that should allow the study of the expression of a particular pattern of calcium channels,  $[Ca^{2+}]_c$  signals, and exocytosis in more physiological conditions.

### III. MODULATION OF CHROMAFFIN CELL CALCIUM CHANNELS

Dunlap and Fischbach (133) were the first to report that the exogenous application of norepinephrine, GABA, or serotonin onto the surface of chick sensory neurons inhibited their  $Ca^{2+}$  conductance. This observation was

soon corroborated in rat sympathetic neurons (132, 133, 156) and was demonstrated to affect HVA, but not LVA, calcium channels (115). The inhibition of the current was associated with many neurotransmitters, receptors, and neurons (79, 120, 165, 197) and was shown to be a membrane-delimited mechanism that was directly coupled to G proteins (122, 195, 203, 211, 235, 399).

#### A. Calcium Channel Current Facilitation and Voltage Dependence of Their Modulation Is a G Protein-Linked Membrane-Limited Phenomenon

Marchetti et al. (257) first observed that the neurotransmitter-mediated inhibition of calcium channel currents was modulated by voltage. Dopamine slows down HVA channel activation, and the effect is stronger at more negative membrane potentials. Another intriguing observation has been that strong depolarizing prepulses augment, instead of produce, the expected voltage-dependent channel inactivation of the calcium channel current induced by milder depolarizing test pulses, an effect that is called "facilitation" (143). We believe that both observations have the same underlying mechanism, a membrane-delimited G protein-mediated inhibition of calcium channels that is relieved by voltage. Figure 2 schematically

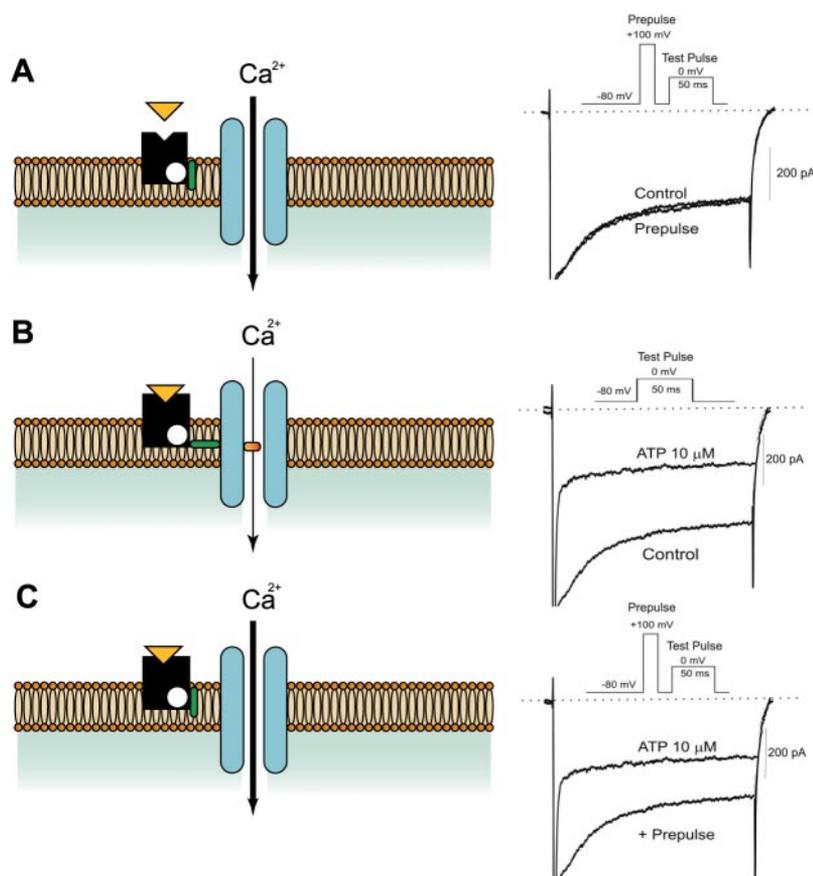


FIG. 2. The mechanism of calcium channel inhibition by G proteins. In resting conditions, G proteins are not coupled to calcium channels, and when channels open, a large amount of  $Ca^{2+}$  flows through them (A). When ATP binds to its purinergic receptor, G protein couples to the channel, slowing down the current activation and decreasing its peak amplitude (B). Application of a strong positive depolarizing prepulse uncouples the G protein from the channel, and the current recovers its control profile (facilitation; C).

shows the G protein-mediated effects of exogenous neurotransmitters on calcium channel currents and the voltage-dependent facilitation by prepulses of the currents. The typical HVA current shows fast activation and meager inactivation, if any (Fig. 2A). When a neurotransmitter binds to its receptor, i.e., ATP in chromaffin cells, it activates a G protein that couples negatively to the calcium channel, promoting a process of slow activation, and the peak current decreases by ~50% (Fig. 2B). When a prepulse protocol is applied in the absence of a neurotransmitter, the normal "test pulse" current is little affected; however, in the presence of a neurotransmitter, i.e., ATP in chromaffin cells, which can slow the activation and inhibition of the current, the prepulse changes the structural conformation of the G protein coupled to the calcium channels and converts any current into another with fast activation and higher peak (facilitation; Fig. 2C). Thus the greater the inhibition of the current produced by a given neurotransmitter, the greater the facilitation by the prepulses. Thus the facilitation depends then on the degree of inhibition of the current, and this inhibition may depend on the secretory activity of the studied cell (autocrine modulation) or neighboring cells (paracrine modulation). These mechanisms will be described in the next sections.

Voltage-induced facilitation may be significant (>80% at +9 mV), but it is usually partial, leaving a variable amount of residual voltage-independent depression. In neurons, voltage-dependent modulation is mostly confined to non-L-type calcium channels (4, 55, 105, 237, 263, 318). Voltage-independent depression has been reported to be associated with N-type channels (250), and P-type channels (364), but mostly with L-type channels in neurosecretory cells (8, 317) as well as peripheral and central neurons (20, 54).

## B. The Chromaffin Cell Is a Good Model to Study Autoreceptor Modulation of Calcium Channels

Pioneering experiments using ATP showed that bovine chromaffin cells were susceptible to neurotransmitter modulation of their calcium channel currents (118, 159). This nucleotide delayed the whole cell calcium channel current activation and inhibited the amplitude of the current. These effects were mediated by P<sub>2y</sub> purinergic receptors through a G protein pathway, as in neurons; they disappear by dialyzing the cells with guanosine 5'-O-(2-thiodiphosphate) (GDPβS), or by pretreating the cell with *Pertussis* toxin, and are mimicked by guanosine 5'-O-(3-thiotriphosphate) (GTPγS). Similar effects by ATP were corroborated 3 years later also in bovine chromaffin cells (111). Opiates (methionine-enkephalin, leucine-enkephalin), through the activation of μ- and δ-receptors, also exert a modulatory action on bovine chromaffin cell

calcium channels, i.e., they slow down channel activation, and decrease the amplitude of the current through a G protein membrane-limited pathway (6).

Various properties make the chromaffin cell a suitable model to study autoreceptor modulation of calcium channels and neurosecretion. 1) Like sympathetic neurons, the chromaffin cell is derived from the neural crest and has all the machinery to manufacture, store, and release catecholamines. 2) The chromaffin cell can be easily isolated from the adrenal glands of various animal species, even in large quantities as in the case of the bovine, and maintained in primary cultures that can survive 1–2 wk. 3) The chromaffin cell expresses various subtypes of voltage-dependent calcium channels that vary considerably with each animal species. 4) The participation of each channel subtype in the control of exocytosis varies markedly with species, stimulation pattern, or cell type (noradrenergic, adrenergic). 5) Chromaffin cells that store epinephrine or norepinephrine also contain a rich cocktail of other chemicals (i.e., ATP, opiates, chromogranins) that are coreleased with the catecholamines. 6) Manipulation of the superfusion system can enhance or decrease the release of these materials, or to direct them to the same patch-clamped cell, or wash materials quickly out from the cell surface. 7) Cells can be cultured in isolation or in clusters to study the influence on the voltage-clamped cells by the materials being released from neighboring cells on the voltage-clamped cells. 8) Simultaneous measurements of calcium channel activity, changes in [Ca<sup>2+</sup>]<sub>i</sub>, and catecholamine release can be monitored, and correlations between these three parameters can be established in the same cell. Although *properties 6–8* can be valid for any cell and culture, chromaffin cells offer the unique advantage that the rich chemical cocktail of their chromaffin vesicles is well known (410), and thus the effects of endogenous chemicals (i.e., ATP and opiates) on calcium channel currents can be studied and characterized.

## C. Flow-Stop Experiments Unmask the Modulation and Facilitation of Calcium Channel Currents

In chromaffin cells (and probably also in several neuronal cell types), the experimental conditions used to record whole cell calcium channel currents under voltage-clamp conditions trigger the exocytotic release of endogenous neurotransmitters; this release is activated by Ca<sup>2+</sup> (or Ba<sup>2+</sup>) entering during the depolarizing test pulses applied to elicit the currents. So, the released neurotransmitter could act backwards onto autoreceptors on the surface of the cell being recorded. If these receptors are coupled to calcium channels via G proteins, they will obviously inhibit the current flowing through them. An experiment to enhance the probability of the

released transmitter combining with its autoreceptors, that increases its concentration near these autoreceptors, consists of stopping the flow of the extracellular solution that bathes the cell being recorded. This manipulation was first used in perfused cat spleen in a pioneering study that was basic to the hypothesis of the modulation of norepinephrine release by presynaptic  $\alpha$ -adrenergic receptors (225). Blockade of these receptors enhances, while their stimulation inhibits, the norepinephrine release induced by low-frequency stimulation of sympathetic nerves. This  $\alpha$ -mediated modulation could not be shown in chromaffin cells (301, 321), even though they are very close relatives of sympathetic neurons. However, we now know that this modulation does exist, but it is associated with purinergic  $P_{2y}$  and opiate  $\mu$ - and  $\delta$ -receptors that act at sympathetic nerve terminals.

In other studies, Doupnik and Pun (128), Albillos et al. (8), and Currie and Fox (111) observed that the rate of activation and the amplitude of  $Ba^{2+}$  currents in bovine chromaffin cells critically depend on the experimental superfusion conditions of the patch-clamped cell. Cell activity under stop-flow conditions (unperfused cell) favors the local rise of secreted products close to the plasmalemma, the subsequent activation of membrane autoreceptors, and the rapid inhibition of spatially localized calcium channels. This tonic inhibition, induced by low-molecular-weight compounds of the vesicle content (i.e., ATP and opiates), coreleased with the catecholamines during application of depolarizing pulses under flow-stop conditions, can be markedly reversed ("facilitated") by strong depolarizing prepulses. The tonic inhibition of the current is also reversed upon resuming the rapid flow over the surface of the cell, which quickly washes the released materials out (Fig. 3).

#### D. Direct Approaches Demonstrate That Endogenously Released Neurotransmitters Modulate Calcium Channels

If ATP and enkephalins modulate calcium channels, and ATP and opiates are co-stored with catecholamines at high concentrations in chromaffin vesicles (410), it would be interesting to test whether the vesicular contents could produce the same effect as the exogenous application of these compounds. Chromaffin vesicles were purified from a bovine adrenal medulla homogenate to prepare a soluble vesicle lysate (SVL). When applied with the extracellular solution onto the surface of a voltage-clamped bovine chromaffin cell, SVL inhibited HVA currents in a concentration- and voltage-dependent manner (8). The modulated current exhibited the same slow activation kinetics as those produced by exogenously applied ATP or methionine-enkephalin; in fact, a mixture of purinergic and opiate receptor blockers antagonized the effects of

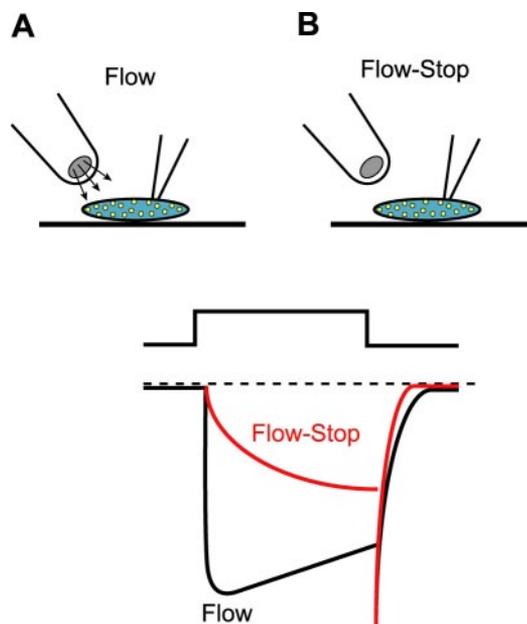


FIG. 3. Drawing illustrating the technical approaches followed to study the regulation of calcium channel currents by endogenously released materials. *A*: fast superfusion conditions. *B*: flow-stop conditions.

SVL. Also, depolarizing prepulses evoked a strong current facilitation in the presence of SVL, indicating that the facilitated current was originated in the suppression of the tonic inhibition of calcium channels by ATP and opiates secreted during cell stimulation.

#### E. Manipulation of the Rate of Secretion: Modulation of Calcium Channels in Cell Clusters

If a procedure could be found to block exocytosis without suppressing calcium channel currents, the currents would not be inhibited and then facilitated as they switch from superfusion to flow-stop. This was true in voltage-clamped bovine chromaffin cells dialyzed with tetanus toxin, which does not affect calcium channel current, but does hydrolyze synaptobrevin and block exocytosis (A. Albillos and A. G. García, unpublished results).

However, there are conditions that can enhance exocytosis and the concentration of materials available to the cell in which the calcium channel currents are being recorded. For instance, the use of  $Ca^{2+}$  instead of  $Ba^{2+}$  as charge carrier can drastically change the secretion rate, since  $Ba^{2+}$  is a more powerful secretagogue than  $Ca^{2+}$  (192) and is poorly chelated by the EGTA that is present in the intracellular solution. Thus, in the presence of 10 mM  $Ca^{2+}$ , stopping the flow had effect on modulation of the current; however, when the same cell was bathed with 10 mM  $Ba^{2+}$ , flow-stop caused a drastic slowing down of current activation and a large decrease in peak amplitude (192).

When the cell whose calcium channel currents are being explored belongs to a cell cluster (Fig. 4*B*), strong inhibition and prepulses facilitation of the whole cell current are observed, if 10 mM  $Ba^{2+}$  (but not  $Ca^{2+}$ ) is used (Fig. 4, *C* and *D*). This result was clearly obtained in human (162) as well as in bovine chromaffin cell clusters (192).  $Ba^{2+}$  (but not  $Ca^{2+}$ ) induced a powerful secretory response (395) in the unpatched, surrounding cells; the secreted materials would be reaching the patch-clamped cell at high concentrations, thereby causing visible modulatory effects on the calcium channel current (Fig. 4*D*). The experiments shown in Figure 4, *E* and *F*, were performed in cells superfused with 10 mM  $Ca^{2+}$  (instead of  $Ba^{2+}$ ).  $Ca^{2+}$  requires  $K^+$  depolarization to activate exocytosis in chromaffin cells. So, in Figure 4*F*, secretion was stimulated in a cell cluster using a brief ap-

plication of a  $K^+$ -rich solution ( $70K^+$ , 1 s); under these conditions, the  $Ca^{2+}$  current that was being recorded from the patch-clamped cell within the cluster activated with a slow kinetics and halved its amplitude; this indicates that the voltage-clamped cell is under the modulatory influence of materials released from neighboring cells by the  $K^+$  pulse. Modulation of the  $Ca^{2+}$  current (after the  $K^+$  pulse) is not seen in an isolated cell (Fig. 4*E*). Experiments on cell clusters were performed by Callewaert et al. (69) in bovine chromaffin cells; they concluded that calcium channel currents were regulated through the release of protons, rather than ATP or opiates. Cell clusters are more similar than isolated cells to intact adrenal medullary tissue, in which secretory materials from neighboring cells can impinge on a given cell and strongly modulate its electrical properties and

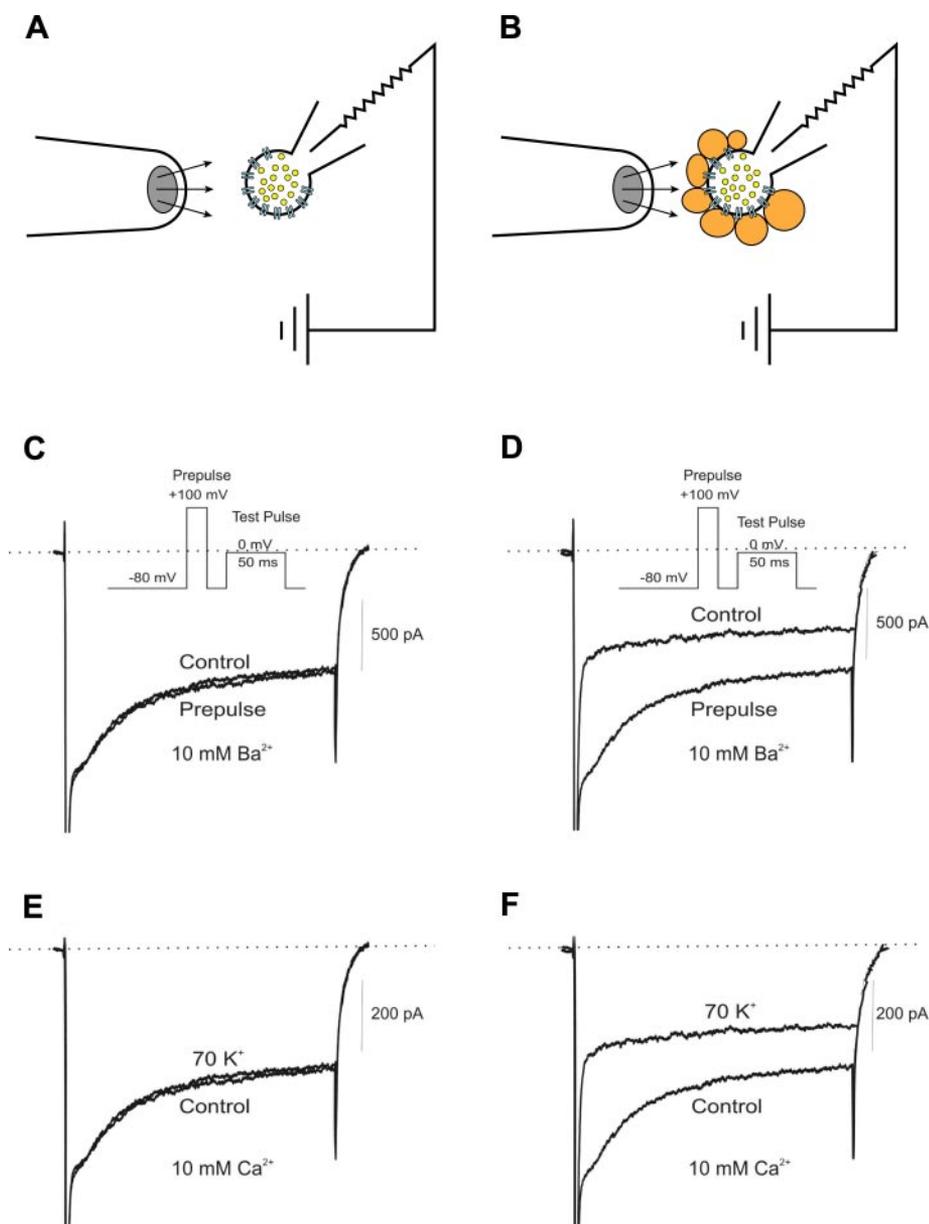


FIG. 4. Modulation of calcium channel currents in a single chromaffin cell (*A*, *C*, *E*) or in a cell immersed in a cell cluster (*B*, *D*, *F*). Cells *C* and *D* were superfused with an extracellular solution containing 10 mM  $Ba^{2+}$ ; cells *E* and *F* were superfused with 10 mM  $Ca^{2+}$ . Before the test pulse to 0 mV (with or without a prepulse to +100 mV), a 70 mM  $K^+$  solution was applied during 1 s ( $70 K^+$ ). See text for further details. [Adapted from Gandia et al. (162) and Hernandez-Guijo et al. (192).]

secretory activity. Hence, studies in adrenal medulla slices should provide further insight into these autocrine-paracrine modulatory mechanisms.

### F. Modulation of L-Type Versus Non-L-Type Calcium Channels: Some Conflicting Points

Because chromaffin cells express four types of HVA calcium channels (L, N, P/Q, and R), the question is whether all of them are equally modulated by endogenously released ATP and opiates. It is interesting that L- and non-L-type calcium channels in bovine chromaffin cells seem to be modulated by opioids through different mechanisms (6). L channels are modulated mostly through a voltage-independent mechanism (no facilitation was observed associated to L channels); non-L channels appear to be inhibited through a voltage-dependent pathway. Thus nifedipine blocks the voltage-independent component with high selectivity (60% block of the voltage-independent component, 5% block of the voltage-dependent component). While  $\omega$ -conotoxin MVIIC (a blocker of N and P/Q channels) completely blocks the voltage-dependent component. The voltage-dependent component was distributed 58% from N channels and 42% from P/Q channels. These data are similar to those obtained by Currie and Fox (111) also in bovine chromaffin cells when they use ATP to modulate the calcium channel currents: N-type channels contributed 69%, and P/Q channels 47%, to the voltage-dependent modulation.

Contrary to our results, a series of papers from the laboratory of Aaron Fox and co-workers (25–28, 30–32) found that prepulse facilitation of calcium channel currents was entirely blocked by nisoldipine, a blocker of L-type calcium channels, and G proteins were not necessary for voltage-dependent facilitation recruitment (32). They argued that “the expression of the facilitation L channel is strongly dependent on the age of the animals from which chromaffin cells are prepared” (25), suggesting that L-type channel facilitation is a property of chromaffin cells from 10- to 12-wk-old young calves. Thus facilitation would be associated with an L-type channel that does not usually contribute to normal  $\text{Ca}^{2+}$  currents.

We believe that the reasons behind these conflicting results may have different origins living on the complexity of the chromaffin cell system, the autocrine/paracrine nature of calcium channel modulation (8, 128, 159), and a possible coupling between channel subunits and second messenger pathways affecting the up- or downregulation of calcium channel subtypes (197). We would like to stress that the membrane-delimited pathway of calcium channel modulation by neurotransmitters in neurons (197) is also a well-established phenomenon in chromaffin cells; it is highly reproducible and has been observed by six independent laboratories at the macroscopic (5, 6,

8, 111, 128, 159, 226) and single-channel level (77). A study in rat chromaffin cells (201) describes a voltage-dependent facilitation of L-type channels that deviates significantly from that observed in Fox’s laboratory with bovine cells. In the rat, facilitation associated with L-type channels contributes to 6% of the total current at most, and it is insensitive to  $\text{D}_1$  dopamine agonists and to protein kinase A (PKA) activation, and is short lasting rather than persisting for seconds.

Modulation of chromaffin cell L-type calcium channels has been extensively studied in the laboratory of Emilio Carbone during the last years (see Ref. 42 for a review). Among the many modulatory pathways, two are of interest because of their autocrine nature: a G protein-dependent inhibition and cAMP/PKA-mediated potentiation (120, 121). In chromaffin cells both pathways are activated by autoreleased neurotransmitter molecules and produce opposite effects of similar magnitude (85). The inhibition is complete within seconds and is mediated by PTX-sensitive G proteins coupled to  $\text{P}_{2\text{Y}}$ -purinergic,  $\mu$ - $\delta$ -opioid,  $\alpha_2$ - and  $\beta_2$ -adrenoceptors (6, 8, 73, 76, 159, 190, 227) while the potentiation is triggered by  $\beta_1$ -adrenergic receptors and occurs slowly over a few minutes through the activation of a cAMP/PKA pathway, which may act at distant sites from receptors (218, 333). The increase of  $\text{Ca}^{2+}$  entry elicited by PKA activation also contributes to the increase of secreted vesicle’s quantal size (74, 253).

An interesting recent finding relates to the expression of two L-channels subtypes in chromaffin cells,  $\alpha_{1\text{C}}$  and  $\alpha_{1\text{D}}$  (42, 169, 171, 409). These two channel types are not easily distinguishable on the basis of their affinities for dihydropyridines (Table 1) or their biophysical properties (i.e., single-channel conductance, mean open and mean closed times). The only parameter that appears to be significantly different between the two channel types is the ranges of their activation voltage, which shifts towards more negative potentials by 20–25 mV for the  $\alpha_{1\text{D}}$ -subunit (230, 415). Biophysical data at the single-channel recording level suggest that most of the functional L channels in chromaffin cells are probably associated with the  $\alpha_{1\text{C}}$ -subunit, in both bovine (74, 76) and rat chromaffin cells (42). According to the available data, it is likely that the G protein-mediated inhibition and cAMP/PKA-mediated potentiation converge on the same channel type, again probably the  $\alpha_{1\text{C}}$ . A voltage-independent mechanism for autocrine inhibition of P/Q-type calcium channel currents that requires Src family kinase activity has also been reported (401).

### G. Physiological Relevance of Calcium Channel Modulation

The effects of neurotransmitters on  $\text{Ca}^{2+}$  currents and exocytosis have been studied in bovine chromaffin

cells by combining membrane capacitance measurements and whole cell current recordings. Extracellular ATP markedly inhibits L-, N-, and P/Q-type currents and exocytosis in a parallel manner; the ATP does not alter the  $\text{Ca}^{2+}$ -dependent fusion of vesicles to the plasmalemma or the vesicle supply to the release sites, suggesting that the inhibitory effects of ATP on exocytosis are primarily associated with calcium channels (320, 382). In rat chromaffin cells, ATP inhibits exocytosis either by depressing  $\text{Ca}^{2+}$  currents (L, N, P/Q) or by directly acting on the secretory machine through a  $\text{Ca}^{2+}$ -independent pathway (236). In rat chromaffin cells, where L-type calcium channels dominate, the cAMP-permeant analog pCPT-cAMP potentiates both the L current and depolarization-evoked secretion; however, the current increase accounted for only 20% of the total secretory response. cAMP doubled the size of the readily-releasable pool of vesicles in these rat cells (75). It is also interesting that chronic stimulation of  $\beta$ -adrenergic receptors and cAMP recruit T-type calcium channels that functionally control secretion in rat chromaffin cells (290).

Slowing down current activation, ATP plus opiate inhibition of peak calcium channel currents should cause profound alterations in  $\text{Ca}^{2+}$  entry, in local  $[\text{Ca}^{2+}]_c$  transients at exocytotic subplasmalemmal sites, and in the rates of norepinephrine and epinephrine release. These effects may constitute the basis for the fine tuning of the quantity of adrenal medullary catecholamines delivered to the circulation during stressful incidents. This fine regulation is absolutely necessary to prevent massive uncontrolled release of catecholamines that could lead to a hypertensive crisis, arrhythmias, and myocardial damage. Catecholamines are required quickly and at the appropriate concentrations, by target organs needing to adapt to mental or physical stress, or in emergencies requiring a fast "fight or flight" response by the entire body. But the catecholamines stored in both adrenal glands can kill the animal, if they are suddenly released into the circulation; a precise and efficient control of their rate of secretion is needed. The data reported here would explain the regulation of catecholamine release to the circulation as follows (Fig. 5).

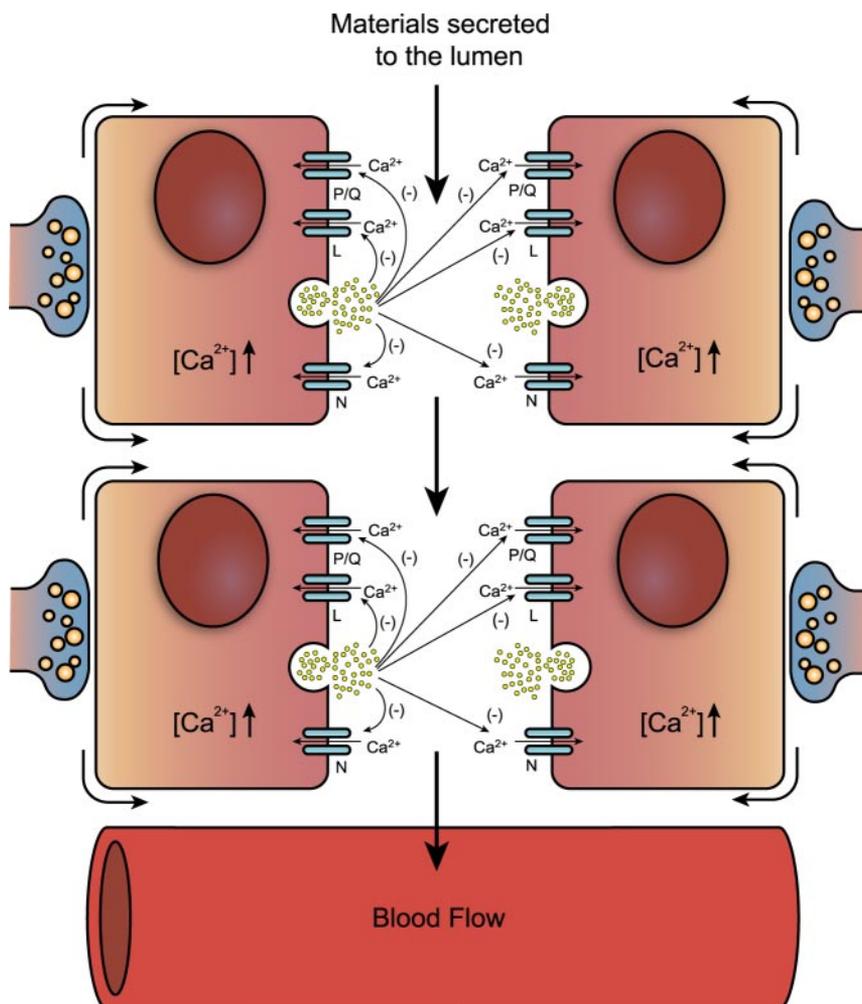


FIG. 5. Scheme showing our present view on how adrenal medullary calcium channels and catecholamine release are modulated by an autocrine/paracrine feedback mechanism activated by the ATP and opiates coreleased with norepinephrine and epinephrine.

Chromaffin cells adopt a columnar disposition around a small capillary vessel in the adrenal medulla of mammals. The cell secretory surface is exposed to the blood concentrations of secretory materials released from the cells (i.e., catecholamines, ATP, opiates). In resting conditions, or during mild stimulation of splanchnic nerves, the concentrations are obviously low, and thus the purinergic and opiate receptors are not stimulated and the calcium channels are not receiving modulatory stimuli (autocrine/paracrine inhibition). During a sudden stress, the splanchnic nerves release acetylcholine and provoke cell depolarization in response to nicotinic receptor activation, calcium channel opening,  $\text{Ca}^{2+}$  entry, catecholamine release, and a concomitant elevation of opiates and ATP in the immediate vicinity of the cell secretory surface. Released materials then activate  $\text{P}_{2\text{y}}$  and  $\mu$ - and  $\delta$ -receptors and inhibit calcium channels,  $\text{Ca}^{2+}$  entry, and secretion producing a tonic inhibition. If the stress situation persists, and more catecholamine secretion is required, repetitive acetylcholine-evoked action potentials will relieve the tonic inhibition. The voltage-dependent facilitation of non-L-type channels from their resting inhibition promote the extracellular  $\text{Ca}^{2+}$  entry required for the massive release of catecholamines during the “fight or flight” response, or during severe conditions, such as asthma crisis, acute myocardial infarction, anaphylactic shock, or heart failure. However, even in these extreme conditions, nonselective voltage-independent inhibition of calcium channels will maintain a basal level of feedback control to limit hormonal oversecretion. Thus the rate of catecholamine release will be fine tuned at each moment by the interplay between the degree of  $\text{P}_{2\text{y}}$ ,  $\mu$ -, and  $\delta$ -autoreceptor tonic activation by endogenous agonists coreleased with the catecholamines, and the activation of nicotinic receptors on the surface of chromaffin cells.

It is noteworthy that although the modulation of the different calcium channel subtypes has been thoroughly studied in chromaffin cells, the functional consequences of this modulation on  $\text{Ca}^{2+}$  signals and exocytosis is very poorly documented. Simultaneously measuring the “modulated”  $\text{Ca}^{2+}$  currents through a given channel subtype, the ensuing  $[\text{Ca}^{2+}]_{\text{c}}$  increase, and the secretory activity in the same cell using both capacitance and amperometric techniques should provide very interesting data and should provide physiological significance if done in cell clusters or adrenal slices as well.

#### IV. CONTRIBUTION OF EACH CALCIUM CHANNEL SUBTYPE TO TRIGGERING EXOCYTOSIS IN CHROMAFFIN CELLS OF DIFFERENT ANIMAL SPECIES

As described above, different calcium channel subtypes are found on the plasmalemmal membrane of chromaffin cells. This coexistence raises the question as to

whether all of the channel types participate in the control of exocytosis and how their density and properties would condition their participation if any. Furthermore, the presence and proportion of the various calcium channels subtypes varies widely between the animal species (Fig. 1). Therefore, catecholamine secretion from these cells will presumably be controlled differently, in accordance with the calcium channels expressed by the cells. Here, we will review how catecholamine secretion is controlled in different animal species and how some subtypes of calcium channels are more directly implicated in the control of exocytosis. It is important to emphasize that, depending on the type of stimulus used (i.e.,  $\text{K}^{+}$  depolarization, acetylcholine, step depolarizations, action potentials), one type of channel may be more favored over another in secretion. For this reason, the type of stimulus used is indicated in each of the following subsections.

##### A. Cat Chromaffin Cells

The  $\text{K}^{+}$ -evoked secretion of catecholamines is effectively blocked in a concentration-dependent manner by DHPs and other drugs acting on L-type calcium channels like verapamil and diltiazem (161). Measuring differential secretion of epinephrine and norepinephrine, Cárdenas et al. (81) demonstrated that secretion of both amines is completely blocked when it is induced by either high  $\text{K}^{+}$  or the nicotinic agonist dimethylphenylpiperazinium (DMPP). Initially, these data indicated that an L-type channel controlled secretion in these cells. But, Albillos et al. (5) showed that cat chromaffin cells also contained  $\omega$ -conotoxin GVIA-sensitive channels in addition to the L-type channels. It was then demonstrated that HVA L and N calcium channels in cat chromaffin cells were present in an approximate proportion of 50–50% and that the increase in  $[\text{Ca}^{2+}]_{\text{c}}$  induced by short (10 s) depolarizing pulses (70 mM  $\text{K}^{+}$ ) could also be reduced 44% by flunaridol and 43% by  $\omega$ -conotoxin GVIA. In a perfused adrenal gland or isolated cat chromaffin cells, catecholamine release induced by 10-s pulses of 70 mM  $\text{K}^{+}$  was blocked by more than 95% with flunaridol and only 25% with  $\omega$ -conotoxin GVIA. These results show that although  $\text{Ca}^{2+}$  entry through both channels (N and L type) leads to similar increments of the average  $[\text{Ca}^{2+}]_{\text{c}}$ , the control of the  $\text{K}^{+}$ -evoked catecholamine release response in cat chromaffin cells is dominated by the  $\text{Ca}^{2+}$  entering through L-type calcium channels (247). However, more recent data suggest that when exocytosis is measured using capacitance techniques, and the membrane potential is held at  $-80$  mV, the N-type channels also contribute to exocytosis (G. Arroyo, M. Aldea, A. Albillos, and A. G. García, unpublished data). It may be that previous experiments using cell populations or intact cat adrenal glands (81, 161) and long-duration (seconds) depolarizing stimuli inactivated the N-type calcium channels.

## B. Bovine Chromaffin Cells

K<sup>+</sup>-evoked catecholamine secretion from bovine chromaffin cells is greatly potentiated in the presence of the DHP L-type channels agonist BAY K 8644; the rise in secretion parallels the increase in <sup>45</sup>Ca uptake (168). Ceña et al. (84) showed that nitrendipine completely blocked catecholamine release ([<sup>3</sup>H]norepinephrine) in bovine chromaffin cells stimulated with high K<sup>+</sup>. These results do not agree with those obtained by other authors who found that in bovine chromaffin cells DHP did not block more than 40–50% of the secretion (163, 217, 303). The differences may be based on different stimulation patterns and the use of cultured chromaffin cells, fast-superfused cell populations, or the intact perfused adrenal gland.

When it was possible to selectively block specific subtypes of calcium channels with toxins, it was demonstrated that these cells contain other calcium channel subtypes besides L, i.e., N and the P/Q type (9, 43, 57, 58, 157).  $\omega$ -Conotoxin GVIA was ineffective or just barely effective in blocking K<sup>+</sup>-evoked catecholamine secretion (26, 28, 129, 144, 217, 249, 303) in bovine chromaffin cells.

Reports on the contribution of P-type calcium channels to catecholamine secretion vary in the literature. Granja et al. (180) showed that catecholamine secretion induced by high K<sup>+</sup> is not affected by  $\omega$ -agatoxin IVA (100 nM); nevertheless, when secretion was activated by nicotine, the  $\omega$ -agatoxin significantly decreased catecholamine release by 50%. Thus Granja et al. (180) conclude that  $\omega$ -agatoxin IVA could also affect the nicotinic receptor. Duarte et al. (129) show that FTX decreases K<sup>+</sup>-evoked norepinephrine release to 25% and epinephrine release to 39% of the control levels; the combination of FTX plus nitrendipine further decreases norepinephrine and epinephrine release to 12 and 24% of the control levels. Baltazar et al. (44) showed that bovine chromaffin cells contain two types of  $\omega$ -agatoxin IVA-sensitive calcium channels and that the contribution of the P-type channels to secretion is higher at low depolarization levels.

The L-N-P-insensitive portion of catecholamine release in bovine chromaffin cells seems to be  $\omega$ -conotoxin MVIIC sensitive. López et al. (249) observed that catecholamine release from superfused bovine chromaffin cells (stimuli: 70 mM K<sup>+</sup> for 10 s) was inhibited 50% by DHP flunarizine (3  $\mu$ M).  $\omega$ -Conotoxin MVIIC (3  $\mu$ M) also reduced the secretory response by 50%. The combination of flunarizine with  $\omega$ -conotoxin MVIIC completely abolished secretion. On the other hand, these authors also demonstrated that  $\omega$ -conotoxin GVIA and  $\omega$ -agatoxin IVA have no effect on secretion. These results strongly suggest that secretion in these cells is predominantly controlled by Ca<sup>2+</sup> entering through the L- and Q-type calcium channels.

Further studies performed by Lara et al. (233) suggest that Q-type channels are coupled more tightly to active exocytotic sites than are the L-type channels. This hypothesis was suggested by the observation that the external Ca<sup>2+</sup> that enters the cell through a calcium channel, located near chromaffin vesicles, will saturate the K<sup>+</sup> secretory response at both extracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>e</sub>), i.e., 0.5 and 5 mM. In contrast, Ca<sup>2+</sup> entering through more distant channels will be sequestered by intracellular buffers and will therefore not saturate the secretory machinery at a lower [Ca<sup>2+</sup>]<sub>e</sub>.

## C. Rat Chromaffin Cells

DHPs block secretion in perfused rat adrenal glands in a concentration-dependent manner. The magnitude of this blockade is related to the type of stimuli employed to induce secretion. The DHP isradipine can fully block secretion when the stimuli used are K<sup>+</sup> or nicotine. In contrast, when electrical field stimulation is used, the DHPs can only obtain a partial blockade and the inhibition is frequency dependent (248).

Measuring Ca<sup>2+</sup> currents and capacitance, Kim et al. (223) have shown that  $\omega$ -conotoxin GVIA (1  $\mu$ M) blocks 40%, and nifedipine around 60% of the total capacitance increase in rat chromaffin cells. Therefore, secretion in these cells would be controlled by L- as well as by N-type calcium channels. Other recent studies suggest that secretion in rat chromaffin cells is supported by all the available calcium channels (74, 173).

The role of each calcium channel subtype in secretion has also been studied in intact whole adrenal glands from rats. Secretion evoked by depolarizing stimuli like high K<sup>+</sup> was strongly inhibited (80%) by L-type calcium channel blockers, whereas acetylcholine-evoked responses were inhibited equally by either flunarizine or  $\omega$ -conotoxin MVIIC (337). Electrical field stimulation of intact glands releases acetylcholine and other cotransmitters from the splanchnic nerves (397). Under these conditions, N-type calcium channels seem to contribute to the maintenance of the secretory responses, probably through acting on presynaptic channels at the splanchnic nerve terminals (337). In rat chromaffin cells treated with cAMP, a "low-threshold" exocytotic response was triggered at very low depolarizations; this unusual secretory response is associated with the  $\alpha_{1H}$ -subtype of calcium channels (173).

## D. Dog Chromaffin Cells

Kimura et al. (224) have studied the effects of  $\omega$ -conotoxin GVIA and L-type calcium channel blockers (nifedipine and verapamil) on catecholamine release in anesthetized dogs. Catecholamine release into the blood-

stream was induced either by electrical stimulation of the splanchnic nerve or by intra-arterial injection of acetylcholine. Administration of 0.4  $\mu\text{g/ml}$  of  $\omega$ -conotoxin GVIA reduced catecholamine secretion by 30% in response to the electrical stimulation used, and nifedipine or verapamil had no effect under these experimental conditions. However, when catecholamine release was induced by acetylcholine,  $\omega$ -conotoxin GVIA blocked secretion by  $\sim$ 50% and nifedipine also reduced it by 50%. These results suggest that N- and L-type calcium channels contribute to the release of catecholamines in the dog adrenal gland. To our knowledge, there is no available patch-clamp study that determines the subtypes of calcium channels expressed by dog chromaffin cells.

### E. Mouse Chromaffin Cells

Simultaneous recordings of  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ ) and change in membrane capacitance ( $\Delta C_m$ ) in isolated mouse chromaffin cells indicate that exocytosis is proportional to the relative density of each calcium channel subtype: 40% L, 34% N, 14% P/Q, and 11% R (12). This is in line with some of the observations reported above on bovine (139, 251) and rat chromaffin cells (74). In  $\alpha_{1A}$ -deficient mice, the L component of  $I_{\text{Ca}}$  rose to 53% while the P/Q channel contribution disappeared; the N and R contributions were similar (12). This indicates that under the perforated-patch configuration, the secretory response elicited by 200-ms depolarizing pulses is a strict function of the amount of  $\text{Ca}^{2+}$  entering the cell, by whatever calcium channel subtype, L, N, P/Q, or R. In addition, it seems that any calcium channel type colocalizes with the secretory machinery in a similarly random manner and shows the same relative efficacy in activating exocytosis, depending on its density (12). This conclusion differs from that obtained in another study in acutely isolated adrenal mouse slices. In this later study (11), the proportion of channel subtypes differs from that obtained in cultured mouse chromaffin cells (191), i.e., 27% L, 35% N, 22% P, 23% Q, and 22% R. It is curious, however, that the R channels (22% of total current) control as much as 55% of the rapid secretion. Thus Albillos et al. (11) conclude that "R-type calcium channels in mouse adrenal slice chromaffin cells are in close proximity to the exocytotic machinery and can rapidly regulate the secretory process."

### F. Specialization of Calcium Channel Subtypes

The expression of various subtypes of calcium channels in neurons (299) and chromaffin cells (166) poses the interesting question of their specialization in controlling different cell functions. The particular segregation by the different calcium channel subtypes to dendrites, axon

terminals, or somata facilitates their specialization for specific functions in various neuronal cell types. For instance, N- and P/Q-type channels, which are predominantly found along the length of apical dendrites and in axon terminals that synapse on dendrites (404), control the release of various neurotransmitters (407). In contrast, the L-type channels located on proximal dendrites and neuronal cell bodies (2, 372, 403–405) have been associated with the regulation of gene expression and enzyme activity in cortical and hippocampal neurons (39, 114, 137, 280, 406).

In contrast to neurons, studies on the specialization of calcium channel subtypes in cultured spherical chromaffin cells gave unclear results. With the use of  $\text{K}^+$  depolarization and selective blockers, it was concluded that  $\text{Ca}^{2+}$  entry through L- and P/Q-type channels controlled the  $\text{K}^+$ -evoked catecholamine release responses; in contrast,  $\text{Ca}^{2+}$  entry through N-type channels ( $\sim$ 30% of the total) did not contribute to exocytosis in superfused bovine chromaffin cells (249). Also, in bovine cells, using low and high  $[\text{Ca}^{2+}]_e$ , it was concluded that P/Q-type channels were closer to the secretory machinery than the L-type channels (233) (Fig. 6A). With the study of differential secretion of norepinephrine and epinephrine in separate populations of bovine chromaffin cells, it was also found that L-type channels dominate exocytosis in norepinephrine-containing cells while P/Q-type channels did so in epinephrine-containing cells (246). In the perfused cat adrenal gland and in isolated cat chromaffin cells, the  $\text{K}^+$ -evoked secretory response is controlled by L-type channels, suggesting a colocalization of these channels with secretory vesicles (247). On the other hand, in the perfused bovine adrenal gland it was found that nisoldipine blocked the acetylcholine-evoked response by 70% while only blocked 30% of the  $\text{K}^+$ -evoked secretion (217). In contrast, electrical stimulation-evoked release was blocked mostly by  $\omega$ -conotoxin GVIA (294). Thus, depending on the pattern of stimulation, the preparation (cultured cells vs. intact gland), L-, N-, or P/Q-type calcium channels might control the secretory response in bovine chromaffin cells to different extents.

The efficacy of the different channels in controlling exocytosis varies with the degree of depolarization and the concentration of external  $\text{Ca}^{2+}$  used in the experiments. There are different examples in the literature that demonstrate this fact. For instance, Turner et al. (380) observed that the efficacies of  $\omega$ -agatoxin IVA and  $\omega$ -conotoxin GVIA to block glutamate release from rat cortical synaptosomes increased when  $\text{Ca}^{2+}$  influx was reduced by decreasing the external concentration of KCl, decreasing the extent of depolarization, decreasing of the external concentration of  $\text{Ca}^{2+}$ , or partially blocking the  $\text{Ca}^{2+}$  influx with antagonist or another. For example, glutamate release was inhibited by  $\omega$ -conotoxin MVIIC with an  $\text{IC}_{50}$  of 200 nM when stimulation of secretion was induced with



nel subtypes, when one tries to sum up the individual inhibitory effects of the toxins, the values obtained are >100%. They suggest that these findings could be explained by the presence of "spare" channels. Under conditions in which the  $[Ca^{2+}]_c$  is saturating for the acceptor, participation of multiple calcium channels might increase the reliability of excitation-secretion coupling, since activation of a single channel will be sufficient to maximize the release probability. This "spare channel" model might describe excitation-secretion coupling under conditions of relatively strong stimulation, such as high-frequency trains of action potentials, or with prolonged depolarizations using increasing concentrations of  $K^+$ . Biochemical modifications (such as phosphorylation), which increase the sensitivity of the  $Ca^{2+}$  acceptor, would also predict an increased probability of release elicited by entry of  $Ca^{2+}$  through a single channel. Under these conditions, the binding affinity of the calcium channel antagonists would be underestimated by their effect on synaptic transmission, since blockade of one of several channels at the active zone would have little or no effect on release. This would produce a rightward shift in the concentration-response relationship relative to the binding curve.

A number of studies performed in voltage-clamped bovine chromaffin cells have also produced contradictory results. For instance, Artalejo et al. (26) measured  $\Delta C_m$  elicited by a train of 10 depolarizing pulses of 50 ms to +10 mV separated by 500 ms (5 s of stimulation) in bovine chromaffin cells; 10 mM  $Ba^{2+}$  (or  $Ca^{2+}$ ) was used as charge carrier. They found that so-called "facilitation" calcium channels (DHP-sensitive L-type channels) contributed 80% of the exocytosis. The authors suggest that "facilitation  $Ca^{2+}$  channels may be closer to the docking and release sites than either of the other two channels" (Fig. 6E).

The delay between action potentials and amperometric release events is considerably shorter (3 ms) when L-type channels are involved (136). The preferential coupling and short delay indicate that L-type channels colocalize with a pool of ready release vesicles (Fig. 6E). Also, mouse chromaffin cells have a small pool of vesicles that dock near calcium channels and rapidly release their content with depolarizations as short as 2 ms (276).

Lukyanetz and Neher (251) also measured  $\Delta C_m$  in response to single 200-ms depolarizing pulses applied to bovine chromaffin cells under the whole cell configuration of the patch-clamp technique, using 60 mM  $Ca^{2+}$  as charge carrier. They could not obtain the facilitation of  $\Delta C_m$  observed by Artalejo et al. (26). In addition, they could not observe a preferential role of any calcium channel subtype (in eliciting exocytosis) either the action of the calcium currents was proportional to the  $Ca^{2+}$  charge, irrespective of channel type. Contrary to Artalejo et al. (26), Lukyanetz and Neher (251) reported that "participation of N-type channels (in exocytosis) is higher than that

of L-type." The P/Q channel contributed little to  $I_{Ca}$  and  $\Delta C_m$ ; this may be due to the fact that under conditions of excess divalent cation [60 mM  $Ca^{2+}$  was used as charge carrier by Lukyanetz and Neher (251)],  $\omega$ -conotoxin MVIIC binds and blocks P/Q channels poorly (10). Ulate et al. (382) studied voltage-clamped bovine chromaffin cells, measuring  $I_{Ca}$  and  $\Delta C_m$  elicited by single 100-ms depolarizing pulses in 10 mM  $Ca^{2+}$ . They found that "all  $Ca^{2+}$  channel types (20% L, 48% N, 43% P/Q) contributed to the secretory response in a manner roughly proportional to the current they allow to pass, thus implying a similar efficacy in triggering catecholamine release." Finally, Engisch and Nowycky (139) found that  $\Delta C_m$  evoked by single-step depolarizations "was strictly related to the integral of the voltage-clamped calcium currents, regardless of the  $Ca^{2+}$  channel subtype." These experiments suggest an uneven distribution of calcium channels in bovine chromaffin cells (Fig. 6F).

Carabelli et al. (73), using cell-attached recordings of bovine chromaffin cells, found that within single patches of 1  $\mu m^2$ , 5 or 6 N-type and P/Q-type calcium channels colocalize with opioid and purinergic receptors. Because hardly any "empty" patches were encountered, the authors conclude that N and P/Q channels, as well as opioid and purinergic receptors, were uniformly distributed in the plasmalemma. Delayed activation of N and P/Q channels upon patch or whole cell depolarizing pulses occurred in 40–50% of the patches. Since some part of the inhibition appears to be tonic and resistant to PTX, the data were taken to imply that secretion occurs in 30–40% of the patches. Thus it seems that vesicles may be preferentially localized in some specific sites but not necessarily coclustered with N and P/Q channels.

Neither depolarizing pulses in the range of milliseconds applied to voltage-clamped cells nor second-long pulses applied to cells with free membrane potential are representative of the physiological conditions in which chromaffin cells are stimulated in situ. The first approach has a time resolution closer to the duration of action potentials triggered by endogenously released acetylcholine (222); however, holding the membrane potential at hyperpolarizing voltages may prevent the voltage inactivation of the N and P/Q, but not L channels (390). The second approach is much shorter, time-wise, but cells keep their "physiological" membrane potential free at the moment that the depolarizing pulse is applied. At the physiological resting membrane potential (61, 142), N and P/Q channels might be partially inactivated in chromaffin cells; thus the role of these channels in exocytosis could be underestimated in favor of L channels when high  $K^+$  is used to depolarize the cells (247, 249).

The real sequence of events leading from stimulus to release of catecholamines at the adrenal medulla is unknown. However, several studies make the following sequence feasible. Acetylcholine depolarizes the chromaffin

cell (124), and this causes the firing of action potentials (221). The action potentials would recruit calcium channels, triggering  $\text{Ca}^{2+}$  entry and exocytosis. However, the various calcium channel subtypes suffer different degrees of inactivation, depending on the  $[\text{Ca}^{2+}]_c$  (193) and on the membrane potential (390). Furthermore, chromaffin cells express small-conductance  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels (24) that will also contribute to the regulation of action potential firing and exocytosis (271, 381). In summary, all these observations suggest that each type of calcium channel could exhibit different efficacies in triggering and controlling the secretory process, and the experimental conditions must be appropriately selected to reveal these differences.

The possible specialization of calcium channel subtypes has been explored in functional aspects other than exocytosis. For instance, in bovine chromaffin cells,  $\text{Ca}^{2+}$  entry through L-type calcium channels, but not N- or P/Q-type channels, causes mitochondrial disruption and apoptosis (72). O'Farrell and Marley (293) observed that the individual blockade of a given calcium channel subtype was not enough to prevent the activation of tyrosine hydroxylase by  $\text{K}^+$  depolarization; however, blocking various calcium channel subtypes abolished the enzyme activation. This was also true for the  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$ -evoked increase in the expression of the exocytotic protein SNAP-25 (170) as well as for ERK phosphorylation, which seems to depend on a critical  $[\text{Ca}^{2+}]_c$  rather than on activation of a given calcium channel subtype (260). We have also found that secretory control by mitochondrial  $\text{Ca}^{2+}$  fluxes critically depends on the ability of  $\text{K}^+$  depolarization to create high  $[\text{Ca}^{2+}]_c$  microdomains, rather than on  $\text{Ca}^{2+}$  entry through a given calcium channel subtype (268).

Particularly intriguing is the possibility of studying specialization by calcium channel subtypes to trigger specific  $\text{Ca}^{2+}$  signals and exocytotic responses in conditions more physiological than those used up to now, i.e., trains of acetylcholine-like action potentials, chromaffin cell clusters, or adrenal slices at  $37^\circ\text{C}$ , with or without the manipulation of  $\text{Ca}^{2+}$  fluxes in intracellular organelles (see sect. VII).

## V. SPATIAL ORGANIZATION OF CALCIUM CHANNELS AND THEIR SECRETORY MACHINE

Active exocytotic zones have been widely documented in nerve terminals. In these zones, calcium channels and exocytotic sites are tightly coupled so that action potentials activate neurotransmitter release and synaptic transmission with great efficacy (see review in Ref. 259). The demand for rapid release is not expected in neuroendocrine cells whose secretory products are directly re-

leased into the bloodstream, from which they can act systemically on many target organs. However, chromaffin cells secrete epinephrine and norepinephrine, two hormones that are responsible for the "fight or flight" response that occurs within seconds of a stressful stimulus; thus the secretion rate from chromaffin cells can be considered intermediate between that of neurons and endocrine cells. In the literature, different experimental findings have been taken as evidence against colocalization of calcium channels and chromaffin vesicles. However, still other experiments are consistent with certain specific geographic colocalizations of a given subtype of calcium channel with the secretory machine. Next we analyze these two classes of evidence.

### A. Action Potentials and Exocytosis

If the vesicles are docked far away from the calcium channels (200–300 nm), a one-to-one relationship between action potential and exocytosis is unlikely and the delay will be long. Such a weak coupling was found when single action potentials of a few milliseconds in duration were elicited (136, 419) in rat adrenal chromaffin cells.

Trains of action potentials invariably induce release in rat chromaffin cells (419). This response strongly depends on spike frequency, with a 4-fold increase in release rate over a 10-fold increase in action potential frequency. The delay between onset of the train and the first amperometric secretory event remained long and variable; additionally, a release event tail of 2-s duration was observed at the end of the burst. Similar results were obtained by Elhamedi et al. (136) in bovine chromaffin cells. They found a threshold frequency of 1 Hz for action potential-dependent release, and release was strongly potentiated when the stimulation frequency increased to 7 Hz. Still, even at this latter frequency, the distribution of latencies between action potentials and amperometric events varied widely, ranging up to 145 ms.

Evoked plateau depolarizations, lasting several hundred milliseconds, are invariably coupled to release of several quanta (419). However, Moser and Neher (277) found a fast release component in response to single action potentials of 2-ms duration, evoked in mouse adrenal slice chromaffin cells *in situ*. This fast secretion component was sensitive to BAPTA but not to EGTA. The vesicles were docked within 30 nm of the calcium channels; the pool measured 42 fF, or around 33 vesicles (276) considering that each vesicle contributes 1.3 fF to the increase in membrane capacitance (393). It will be interesting to identify the channel types involved in the recruitment of this rapidly releasable pool.

## B. Use of Calcium Chelators Suggests the Lack of Colocalization Between Calcium Channels and Secretory Vesicles

The spatial organization of calcium channels and vesicles is frequently studied using  $\text{Ca}^{2+}$  chelators to block the release response. Chelators with a slow  $\text{Ca}^{2+}$ -binding rate (like EGTA) do not interfere with exocytosis at the squid giant synapse; however, BAPTA, which has a  $\text{Ca}^{2+}$  binding rate that is 100 times faster, blocks synaptic transmission at this synapse (1). This experiment suggests a close colocalization of calcium channels and synaptic vesicles. In contrast, at distances greater than 50 nm away from the calcium channel, the  $[\text{Ca}^{2+}]_c$  strongly depends on the presence of  $\text{Ca}^{2+}$  buffers (286); under these conditions, slow buffers like EGTA can interfere with secretion.

In chromaffin cells EGTA reduced the chance of observing amperometric events in perforated patch recordings to 15% (98). EGTA shifts the secretion threshold from 100 million  $\text{Ca}^{2+}$  ions with 100  $\mu\text{M}$  EGTA to 750 million with 1 mM EGTA (343). The delay between the influx of  $\text{Ca}^{2+}$  and the fusion of vesicles is usually longer than 20 ms; this time delay is affected by  $\text{Ca}^{2+}$  buffers and thus suggests that the delay is attributable to  $\text{Ca}^{2+}$  diffusion from its site of entry to the vesicle (98). This indicates that chromaffin granules are docked at a substantial distance from calcium channels; thus the dynamics of  $\text{Ca}^{2+}$  concentration is strongly influenced by  $\text{Ca}^{2+}$  buffers. Computer simulation of  $\text{Ca}^{2+}$  dynamics and release suggests that the slow kinetics of release is due to the fact that the calcium channel and the vesicle are separated by 200–300 nm (228). At this distance, the  $[\text{Ca}^{2+}]_c$  peaks at 10  $\mu\text{M}$  (99). This may explain a persistent secretory response after  $\text{Ca}^{2+}$  entry ends (100). On the basis of these data, it is unlikely that the  $\text{Ca}^{2+}$  channel would form an integral part of the exocytotic machine. These large distances make it very likely that  $\text{Ca}^{2+}$  entry through multiple  $\text{Ca}^{2+}$  channels contributes to release of a particular vesicle.

The experiments with  $\text{Ca}^{2+}$  buffers are best explained with a barrier that reduces diffusion by 3,000 times and is located 130 nm beneath the plasmalemma.  $\text{Ca}^{2+}$  dynamics and spatial  $\text{Ca}^{2+}$  gradients and profiles are better simulated by a shell model rather than a microdomain model (292, 336). Klinghauf and Neher (228) justify the shell model on the basis that  $\text{Ca}^{2+}$  has to diffuse more than 100 nm from the channel. Under these conditions the  $\text{Ca}^{2+}$  has to travel a substantial distance towards the release site, and thus creates a time window where EGTA and BAPTA can act to modify  $\text{Ca}^{2+}$  gradients and exocytosis.

## C. Specialized Zones

Nonhomogeneous distribution of release sites in bovine chromaffin cells has been detected using various methodological approaches. Earlier studies using anti-dopamine  $\beta$ -hydroxylase (DBH) antibodies to mark exocytotic sites suggested a random localization over the entire cell surface (196, 310). Placing 1- $\mu\text{m}$  radius carbon fiber electrodes at different points on the cell surface, Schroeder et al. (341) mapped exocytotic sites at 2  $\mu\text{m}$ , alternating with other silent sites on the surface of bovine chromaffin cells. "Hot spots" of  $\text{Ca}^{2+}$  entry and release were also detected by Robinson et al. (332). With a combination of DBH antibodies and confocal microscopy, DBH had a punctate appearance, suggesting once more that exocytosis can occur in specialized patches (408).

Monck et al. (266) used pulsed laser imaging and visualized hot spots of submembraneous  $\text{Ca}^{2+}$  evoked by 50-ms depolarizing pulses. These hot spots developed relatively slowly (20–50 ms) compared with  $\text{Ca}^{2+}$  domains in the same nerve terminals (243–245). The domain occurred underneath the plasmalemma, within  $<2 \mu\text{m}$ , and extended laterally by several micrometers. Partial or complete submembraneous rings of elevated  $[\text{Ca}^{2+}]_c$  appeared at 50–100 ms after channel opening, and the calcium gradient collapsed 200–400 ms after the pulse began. The peak  $[\text{Ca}^{2+}]_c$  was rather low. This was interpreted as due to a limited  $\text{Ca}^{2+}$  diffusion because of action by immobile calcium buffers (266, 420).

## D. Polarization of Chromaffin Cells

Unlike cultured spherical chromaffin cells, chromaffin cells adopt a columnar or polygonal shape in the intact adrenal medullary tissue. Morphological evidence suggests that the distribution of the various organelles is not completely random, so chromaffin cells seem to be polarized within the adrenal medulla (104). This polarization appears to take place with respect to nerve endings and the nucleus being on one side of the cell, with blood vessels, typically sinusoids, on the other side of the cell (82). In fact, secretory vesicles are more numerous near the sinusoids and endothelial cells and are located more densely in the cell pole that is opposite the nucleus (37).

Functional evidence for this polarization was obtained by Cuchillo-Ibáñez et al. (110). Spherical bovine chromaffin cells were stimulated with high  $\text{K}^+$  resulting in markedly enhanced catecholamine release, which was monitored amperometrically. The fluorescence confocal images with the FMI-43 and DBH antibodies were two to three times more intense at the bottom of the cells, compared with the equatorial or apical planes. The authors attributed the polarization to the release of trophic factors, possibly nitric oxide from endothelial cells that grow

in culture with chromaffin cells (155). These factors might exert trophic influences on the exocytotic machine that polarizes it even in cultured spherical cells. This polarized organization is highly suitable for rapid exocytosis during stress, with nervous stimulation approaching one end of the cell and the catecholamines discharged at the other. Similar studies in adrenal slices should provide additional useful information on the “polarization” of calcium channels and the active secretory sites. Although these findings do not prove the existence of microdomains, they do, however, certainly support the presence of geographic specialization in chromaffin cells.

## VI. CALCIUM MODULATION OF EXOCYTOSIS STEPS

The key role of  $\text{Ca}^{2+}$  in stimulus-secretion coupling was first demonstrated in the chromaffin cell by Douglas and Rubin (126) in the perfused cat adrenal gland stimulated with ACh. The first demonstration that an increase of the  $[\text{Ca}^{2+}]_c$  was a requirement for exocytosis was made by Baker and Knight (41) in bovine chromaffin cells. Another key finding came with the demonstration of  $\text{Ca}^{2+}$  and ATP-dependent priming, which make chromaffin vesicles available for prompt exocytosis (204). The most direct approach to show the relationship between a rise in  $[\text{Ca}^{2+}]_c$  and rapid exocytosis came with the use of photoreleased caged  $\text{Ca}^{2+}$ , which revealed the multiple  $\text{Ca}^{2+}$ -dependent steps of exocytosis (288).

Modulation of stimulus-release coupling may take place either at the calcium channels, where it would modify the amount and rate of  $\text{Ca}^{2+}$  provided to the secretion machine, or else intracellularly, at different, early or late steps of exocytosis, whether vesicle transport, docking, priming, or fusion. We analyze this last aspect here, emphasizing the role of  $\text{Ca}^{2+}$  in regulating the various steps of exocytosis.

### A. Separation of Vesicle Pools by the Analysis of Exocytotic Kinetics

Distinct phases of catecholamine release have been characterized by stimulating single chromaffin cells with flash photolysis of caged  $\text{Ca}^{2+}$ , single depolarizing pulses of increasing length, or repetitive depolarizing pulses (see reviews in Ref. 286).

Four different release readiness pools of secretory vesicles have been described depending on the kinetics, morphology, and regulatory properties (Fig. 7) (188, 189, 395). The immediately releasable pool (IRP), or ready release pool (RRP), contains vesicles that are in the final stage of maturation, just before fusion is triggered by the last  $\text{Ca}^{2+}$ -dependent step. The RRP may be defined by the capacitance response generated by photolysis of caged

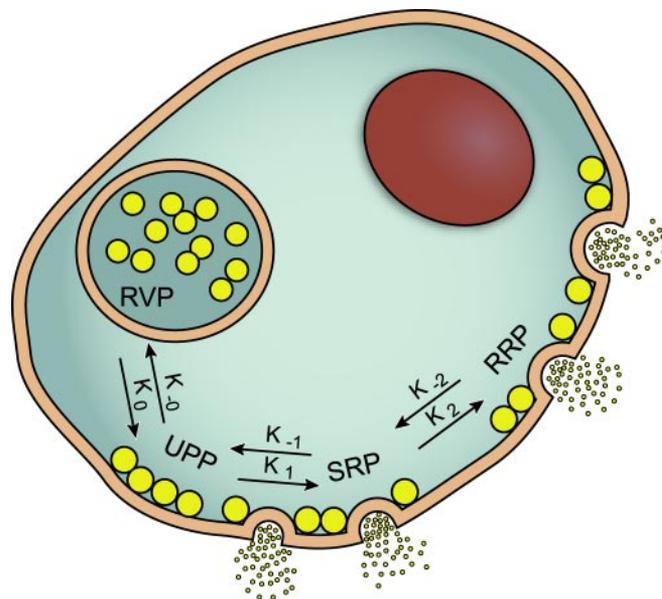


FIG. 7. Scheme showing the different vesicle pools of chromaffin cells, according to their readiness to participate in exocytosis (see text).

$\text{Ca}^{2+}$ , as the asymptotic maximum of release obtained with depolarizing pulses of increasing duration or by applying a paired pulse protocol (175, 277). The RRP is depleted with relatively short delay ( $\sim 100$  ms). The docked vesicle pool (DVP), also called slow releasable vesicle pool (SRP), contains vesicles that are associated with the plasmalemma as a result of the formation of the SNARE complex. Its size can be estimated from the second kinetic component of the release response, which lasts for a few seconds. Its delay may be explained by additional priming steps required by vesicles to become release competent and enter the RRP. An unprimed third vesicle pool (UPP) has also been described (394). The fourth reserve vesicle pool (RVP) contains vesicles that are recruited from the cytoskeleton to become docked at the DVP.

The number of vesicles in each pool is as yet undetermined. Using flash  $\text{Ca}^{2+}$  pulses, Parsons et al. (306) estimated that the DVP contained 830 vesicles. Only 10% of these vesicles are released within 140 ms from the flash; thus the size of the RRP would be  $\sim 100$  vesicles. Vitale et al. (392) found that each bovine chromaffin cell contained 17,000 vesicles, but only 200 of these were located within 50 nm of the plasmalemma (DVP). Steyer et al. (360) followed the movements of GFP-labeled vesicles using microscopic methods. They separated a small RRP within a much larger DVP that turns over through addition of vesicles actively moving to docking sites as well as through the release and reversal of docking.

Separation of vesicle pools has also been achieved by studying regulatory mechanisms. For instance, the slow phases of release taking place on a time scale of seconds

are increased by  $\text{Ca}^{2+}$  and ATP. Thus a  $\text{Ca}^{2+}$ -dependent step mobilizes vesicles from the RVP to the DVP (396). ATP is required to sustain secretion by refilling the RRP (52, 306, 413). This vesicle mobilization from the RVP plays a prominent role in sustaining secretion and may explain the delay observed in various studies. Thus Zhou and Mislner (419) using rat chromaffin cells, and Elhandami et al. (136), using bovine chromaffin cells, reported that the amperometric release of catecholamines could be evoked by action potentials with a delay of up to 2 s.

Depolarizing pulses are much less efficient than  $\text{Ca}^{2+}$  photorelease to trigger exocytosis. Horrigan and Bookman (205) found that a single depolarization evoked the release of 17 vesicles from the RRP in rat chromaffin cells with a time constant of 50 ms. With repetitive pulses at 20 Hz they distinguished two pools. An early pool was depleted with a time constant of 150 ms and closely matched the RRP of 17 vesicles. The second pool was depleted in a few seconds and contained 170 vesicles, a size similar to the DVP as estimated by Vitale et al. (392). A similar size for RRP was found by Gillis et al. (175) in bovine chromaffin cells. It should be noted that the  $\text{Ca}^{2+}$  photorelease protocol produces estimates of the DVP that are much larger than those obtained using depolarizing pulses. This may be due to buffering of  $\text{Ca}^{2+}$  entering through the calcium channels or to slow diffusion of the cation, which would allow only a fraction of the  $\text{Ca}^{2+}$  to reach the competent vesicle target.

A refined model of catecholamine release has been proposed by Voets et al. (394). It is based on the comparison between the vesicle populations released in response to voltage-dependent  $\text{Ca}^{2+}$  entry through calcium channels and flash photolysis of caged  $\text{Ca}^{2+}$ . Flash photolysis of  $\text{Ca}^{2+}$  elicits a biphasic exocytotic burst. The RRP recruited by depolarization corresponds to the vesicle population released during the fast component of the burst. The slow component (slow releasable pool, SRP) remains after RRP depletion and is slightly more activated than the above-described RVP. The SRP (equivalent to the DVP?) can only be directly depleted during prolonged episodes of high  $[\text{Ca}^{2+}]_e$  and may be involved in the rapid resupply of vesicles to the RRP.

## B. Relationship Between $\text{Ca}^{2+}$ Entry and Exocytosis

$\text{Ca}^{2+}$  couple the stimulus to the secretory machine in the neurotransmitter (116) and hormone (126) release processes. The relationship between  $\text{Ca}^{2+}$  and exocytosis is not linear; rather,  $\text{Ca}^{2+}$  cooperate in the process of transmitter release (see Ref. 261 for a review). For instance, at the frog neuromuscular junction, it was first demonstrated that doubling the  $[\text{Ca}^{2+}]_e$  produced an almost 16-fold increase in quantal release (119). This was interpreted as a cooperative interaction between  $\text{Ca}^{2+}$

and transmitter release. Subsequently, a similar relation was found at different synapses (207, 208, 220, 240) and in melanotrophs in the release of large dense-cored vesicles (371).

Other studies have found a linear relation between release and the number of  $\text{Ca}^{2+}$  entering the cell through voltage-activated calcium channels. For instance, the strict linear relation in rat melanotrophs implies that during successive depolarizations (25-step pulses to +10 mV), exocytosis will only decrease in parallel to the inactivation of the  $\text{Ca}^{2+}$  current (255, 256). The same authors also found a constant relation between  $\text{Ca}^{2+}$  entry and capacitance increase, exemplified by the constant efficacy of  $\text{Ca}^{2+}$  in inducing exocytosis, using step depolarizations of 2–40 ms duration or action potentials of 50–200 ms duration. In pituitary nerve terminals there was a linear relation between the  $\text{Ca}^{2+}$  current integral for depolarizing trains at different potentials and the increase in capacitance (342). This was corroborated by Hsu and Jackson (206), who also found a power factor of 1 for the relation between the  $\text{Ca}^{2+}$  current peak and exocytosis, by changing pulse voltage or pulse duration. In bullfrog sympathetic ganglia, exocytosis of luteinizing hormone-releasing hormone from large dense-cored vesicles increased linearly with the amount of the  $\text{Ca}^{2+}$  influx (307). It is interesting that this linearity has also been found in the squid giant synapse, where earlier studies showed that the amplitude of the postsynaptic current also scales to the third or fourth power of the  $[\text{Ca}^{2+}]_e$  (34, 356). In a later study measuring transmitter release, Augustine (33) found that the postsynaptic current scaled linearly with the duration of the action potential. In turn, the duration of the action potential had a linear relationship with the size of the  $\text{Ca}^{2+}$  current and the number of  $\text{Ca}^{2+}$  entering the presynaptic terminal.

In adrenal medullary chromaffin cells, the question of the  $\text{Ca}^{2+}$ -exocytosis relationship has been approached in only a few studies, with varying results. In 1993, Heinemann et al. (189) found that the increase in the  $[\text{Ca}^{2+}]_e$  evoked by caffeine, related to the rate of exocytosis (measured with capacitance techniques), followed a power function with an exponent of 3. Using single depolarizing pulses of increasing length, Engisch and Nowycky (139) found a relation between  $\text{Ca}^{2+}$  entry and exocytosis that followed a power function with variable exponents (between 1.2 and 2); this relation was similar when using trains of depolarizing pulses stimuli (138). Results similar to those of Engisch et al. (138) were obtained by Giovanucci et al. (176) using depolarizing pulses in bovine chromaffin cells; the latter authors also used action potential waveform trains at 5 Hz and found a linear  $Q_{\text{Ca}}-\Delta C_m$  relationship. A recent study on the role of Rab3A in exocytosis in bovine chromaffin cells,  $\text{Ca}^{2+}$  entry, and exocytosis, in cells activated by depolarizing pulse trains, found a strict linear relationship (369). Finally, using de-

polarizing pulses of increasing length (10–150 ms), Carabelli et al. (75) also found a linear relationship between  $\text{Ca}^{2+}$  entry and secretion in rat chromaffin cells.

The main physiological neurotransmitter at the splanchnic nerve-chromaffin cell synapse is acetylcholine (141). By depolarizing the chromaffin cell, acetylcholine triggers action potentials (51, 61, 142); these action potentials are believed to dominate the electrical activity of the chromaffin cell in situ, upon activation of the splanchnic nerve and the release of endogenous acetylcholine (202, 210). The few studies that have analyzed exocytotic responses triggered by trains of simulated action potentials that are reminiscent of those triggered by acetylcholine; however, these studies did not address the question of the quantitative relation between  $\text{Ca}^{2+}$  entry and exocytosis in chromaffin cells (88, 89, 419).

The variability in the quantitative relationship between  $\text{Ca}^{2+}$  entry and exocytosis may be due to the different stimulation patterns used. An example of the variability is provided by a recent study from Corey Smith and co-workers (89). They found that the high-voltage-activated P/Q-type calcium channels dominate the  $\text{Ca}^{2+}$ -dependent exocytotic response in rat chromaffin cells stimulated with action potential trains; however, other channels predominated when square depolarizing pulses were used (89). More puzzling is the fact that in earlier studies, Engisch and co-workers (138, 139) found a relation for  $\text{Ca}^{2+}$  entry and exocytosis fitting a power exponent of 1.5–2, and in a more recent study using the same pattern of square depolarizing pulses, Engisch and co-workers (369) have found a strict linear relationship. Thus it seems that more studies using more physiological patterns of stimulation, i.e., trains of acetylcholine-like action potentials, are needed to know in more detail the  $\text{Ca}^{2+}$  entry-exocytosis relationship in chromaffin cells in culture and in adrenal slices.

### C. Modulation by $\text{Ca}^{2+}$ and Protein Kinase C

An elevation of the  $[\text{Ca}^{2+}]_c$  is not only involved in the late steps of exocytosis but is also involved in earlier steps. Thus the filling of the RRP, measured as recovery from depletion, was enhanced by a mild elevation of the  $[\text{Ca}^{2+}]_c$  induced either by moderate depolarization or by histamine (52, 396). Heinemann et al. (189) incorporated the  $\text{Ca}^{2+}$ -dependent vesicle supply to the RRP and observed that the model adequately explained depression and augmentation of exocytosis.

Many studies have observed that the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) potentiates responses to different secretagogues (53, 63, 67, 68, 109, 229, 316, 335, 350, 366, 367, 391, 392). PMA does not induce exocytosis by itself, but causes disruption of the cortical F-actin network, a barrier that blocks

vesicle movement towards the plasma membrane (66, 92, 93, 351, 375, 376) and doubles the number of vesicles at 50 nm below the plasmalemma as well as the secretion response (392). This effect of PMA is due to PKC stimulation (391, 392), and it is blocked by PKC inhibitors (109, 391). The effect of PMA was mimicked by  $\text{K}^+$  and nicotine, which stimulate  $\text{Ca}^{2+}$  entry and exocytosis. Thus  $\text{Ca}^{2+}$  activates a PKC-mediated phosphorylation step that causes disruption of the cortical actin network. This frees vesicles from the cytoskeletal barrier and allows them to translocate to active secretory sites under the plasmalemma. Gillis et al. (175) confirmed that PMA activates PKC and enhances release. PMA augments the size of the RRP without changing the probability of release from DVP. This conclusion was supported by the fact that PMA selectively increased the amplitude of the exocytotic burst induced by photoreleased  $\text{Ca}^{2+}$ , but it did not modify the kinetics of secretion.

In another study, Smith et al. (349) showed that PMA doubled the RRP by doubling the rate constant for vesicle supply. In addition, reducing the basal  $[\text{Ca}^{2+}]_c$  to 72 nM reduced the RRP to 51 fF, while measuring basal  $[\text{Ca}^{2+}]_c$  to 500 nM raised the RRP to 400 fF. The authors conclude that the increase of RRP may be attributed, at a moderate  $[\text{Ca}^{2+}]_c$ , to direct action by  $\text{Ca}^{2+}$  on the transition rate; however, indirect effects through PKC may contribute to the large effects of a 500 nM basal  $[\text{Ca}^{2+}]_c$ . Later experiments using trains of depolarizing stimuli reached the same conclusion (348). Again, a direct  $\text{Ca}^{2+}$ -dependent action and an indirect, long-lasting effect through PKC were distinguished.

In summary, data from Vitale et al. (392) suggest that PKC promotes recruitment and docking of vesicles, while those of Gillis et al. (175), Smith et al. (349), and Smith (348) indicate a stimulatory action of  $\text{Ca}^{2+}$  and PKC on priming, a postdocking action. It is also intriguing that Munc-13, a protein involved in exocytosis, shows a PMA-dependent but PKC-independent activity (131). On the other hand, Munc-18 phosphorylation in response to PMA treatment of chromaffin cells provides another mechanism for the control of exocytosis (46). Furthermore, PKC-induced phosphorylation of SNAP-25 plays a role in vesicle recruitment (281).

In addition to PKC, other proteins are also targets for  $\text{Ca}^{2+}$ . Thus synaptotagmin, which has been proposed as the final  $\text{Ca}^{2+}$  sensor to trigger exocytosis (361), has been identified in chromaffin cells (295, 377). Nevertheless,  $\text{Ca}^{2+}$ -dependent activator protein for secretion (CAPS) is critical to trigger exocytosis (135), as are other  $\text{Ca}^{2+}$  binding proteins that are enumerated in Table 2.

### D. Modulation of the Final Steps of Exocytosis

Chromaffin cells contain ~10,000–20,000 chromaffin vesicles similar to the large dense-cored vesicles found in

neurons. An important part of our knowledge of exocytosis came from experiments performed in chromaffin cells. These cells were initially chosen for the development of the main techniques for the study of exocytosis at the level of single-cell or single-exocytotic events (patch-clamp capacitance, amperometry, patch-amperometry, or evanescent wave microscopy, to cite only some examples).

It is now widely accepted that exocytosis is a more complex phenomenon than the simple fusion of vesicles storing discrete neurotransmitter packages, a concept derived from the classic quantal theory (117). Recent data suggest that, at least for chromaffin vesicles, second messengers, including  $\text{Ca}^{2+}$ , can modify the quantal size and the kinetics of exocytosis of individual fusion events. In addition, exocytosis does not always mean full-fusion, in as much as several forms of partial release/fusion have so far been described, i.e., “kiss and run,” flickers, and pseudo-flickers (7, 83). Hence, the late stages of exocytosis can be modulated in several ways that include partial versus full fusion, rapid versus slow release, single versus compound fusion, and full versus partially emptied granules.

Another phenomenon observed is the so-called “compound fusion” (19). Secretory vesicles contain most of the putative proteins required for fusion. Under certain conditions, two or more vesicles can fuse before or during exocytosis. This phenomenon seems to be dependent on the  $[\text{Ca}^{2+}]_c$ ; probably it only occurs normally in a limited number of vesicles, but it becomes important under strong stimulation when the  $[\text{Ca}^{2+}]_c$  reaches a high level (56). Compound fusion is a controversial issue. However, it has been demonstrated in mast cells (19) as well as in eosinophils (102). Based on the effect elevating cAMP has on the quantal size, we have proposed the existence of compound fusion in chromaffin cells; we have observed that strong PKA activation increased quantal size of single exocytotic events (56, 253). cAMP has been described to increase calcium channel activity (127). Because the increase in quantal size occurred within seconds, it is unlikely that it would involve an increase in the synthesis or the vesicular uptake of amines. Computer simulation suggested that the increase of quantal size was the result of compound fusion. This explanation gained support from experiments using calcium antagonists; nitrendipine,  $\omega$ -conotoxin MVIIC, and  $\omega$ -conotoxin GVIA reduced the quantal size of individual exocytotic events, thus suggesting the presence of compound fusion under normal stimulation conditions. Conversely, the dihydropyridine agonist BAY K 8644 increased the quantal size of secretory spikes measured with amperometry (56).

The presence of high  $[\text{Ca}^{2+}]_c$  in chromaffin granules raised the question of its possible physiological role.  $\text{Ca}^{2+}$  release by exocytosis is measurable by cyclic voltametry (412); this mechanism will only minimally contribute to

eliminate  $\text{Ca}^{2+}$  from the cell. The presence of inositol trisphosphate ( $\text{InsP}_3$ ) receptors in the membrane of chromaffin granules raised the possibility that vesicular  $\text{Ca}^{2+}$  participate in  $[\text{Ca}^{2+}]_c$  control (418). Although most of vesicular  $\text{Ca}^{2+}$  is associated with the vesicle matrix, the total amount of  $\text{Ca}^{2+}$  in a cell compartment that is very abundant, like chromaffin granules, which occupies nearly 20% of the cell volume, should be taken into consideration. In addition, the  $\text{Ca}^{2+}$  released from chromaffin granules will reach its maximal free concentration near the machinery for controlling vesicle traffic and secretion. Hence, permeable weak bases disrupt the pH gradient through the chromaffin vesicle membrane, thus raising intravesicular free  $\text{Ca}^{2+}$  and free catecholamines (252, 278, 279). The participation of vesicular  $\text{Ca}^{2+}$  in exocytosis was demonstrated by Wightman's group in chromaffin cells using reserpine and tetrabenazine in the absence of external  $\text{Ca}^{2+}$ , and after impairing the mitochondrial and ER  $\text{Ca}^{2+}$  reservoirs (279). We have obtained similar results by dissipating the vesicular pH gradient using the V-ATPase blocker bafilomycin  $\text{A}_1$  (70). Conversely, increasing cytosolic  $\text{Ca}^{2+}$  also facilitates the release of amines at fusion (146).

The increase in vesicular  $\text{Ca}^{2+}$  forces the aggregation of chromogranins (417). In addition, divalent and monovalent cations promote changes in the affinity of mast cell granule matrices (145, 282), and this has caused similar effects on the kinetics of exocytosis in chromaffin cells and mast cells (215, 311). The main conclusion from these studies is that the increase in external  $\text{Ca}^{2+}$  concentrations delayed the diffusion of amines after granule fusion. This action on chromogranin disaggregation could inhibit granule swelling, and be responsible for the shift from exocytosis mode to the kiss-and-run mode when the extracellular  $\text{Ca}^{2+}$  rises (13). Alternatively, very high  $\text{Ca}^{2+}$  could affect the fluidity of lipids of fusion pore membranes.

With the discovery of the main proteins implicated in the exocytotic process (SNAREs), a great effort has been made to address the role of secondary messengers, notably  $\text{Ca}^{2+}$ , in the cascade of the protein-protein interactions. Although a detailed study of the exocytotic machinery exceeds the aim of this review, Table 2 summarizes the effects of  $\text{Ca}^{2+}$  on some proteins that are possibly involved in the different steps of exocytosis.

## VII. CALCIUM ENTRY AND REDISTRIBUTION INSIDE THE CHROMAFFIN CELL: ROLE OF ORGANELLES AND FUNCTIONAL IMPLICATIONS FOR EXOCYTOSIS

The increases of local  $[\text{Ca}^{2+}]_c$  are not only controlled by  $\text{Ca}^{2+}$  entry through a variety of voltage-dependent calcium channels that open during cell activation;  $\text{Ca}^{2+}$

TABLE 2. *Putative Ca<sup>2+</sup> effectors in the releasing process and some related proteins*

Cell Process	Main Discovery	Reference Nos.
Vesicle transport	Ca <sup>2+</sup> is necessary to move vesicles from a reserve pool towards the RRP	349, 396
Actin network disassembly	Scinderin is a calcium-dependent protein that severs actin filaments	334
Last step by the granule towards the membrane	Ca <sup>2+</sup> is needed to transport vesicles to the Ca <sup>2+</sup> -entry sites	47
Docking and priming		
Synaptotagmin I (p65)	Is the main Ca <sup>2+</sup> sensor for fast exocytosis Equilibrates the different vesicle pools Promotes full fusion	40, 64, 147
Synaptotagmin IV	Switch between full and kiss-and-run exocytotic patterns	393, 398
Synaptotagmin III, V, and VII	High affinity for granule exocytosis	362, 363
Synaptotagmin	Interaction with L and R calcium channels	103
Syntaxin	Prolongs or shortens the opening of the fusion pore	177, 186, 187
SNAP25	Modulates the kinetics of exocytosis	106, 174, 353
Synaptobrevin	Modulates the kinetics of exocytosis	174
CAPS	Forces the closing of the fusion pore	135
Myosin II	Decelerates exocytosis	284
Dynamin	Displaces the kinetics of the fusion pore to the closed status	179
Cysteine string protein	Slows the kinetics of exocytosis	178
Cyclin-dependent kinase 5	Reduces the fusion pore conductance	45
Munc18/n-sec 1	Modulates the kinetics of the fusion pore	101, 148
Complexin II	Forces the closing of the fusion pore	22

RRP, ready release pool.

sequestration into and Ca<sup>2+</sup> release from the ER and from mitochondria also control the local [Ca<sup>2+</sup>]<sub>c</sub>. We have learned only recently these organelles play a crucial role in the control of the local [Ca<sup>2+</sup>]<sub>c</sub> transients occurring at subplasmalemmal sites during physiological cell activation; these changes are ultimately responsible for shaping the exocytotic responses upon single or repeated stimulation in chromaffin cells. We summarize here the recent evidence supporting the view that Ca<sup>2+</sup> entering through calcium channels, Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) from the ER, and Ca<sup>2+</sup> uptake into mitochondria form a functional triad to control preexocytotic and exocytotic steps that regulate the release of catecholamines in chromaffin cells.

Ca<sup>2+</sup> signaling becomes quantal at the molecular level because very high Ca<sup>2+</sup> concentrations are required to trigger certain Ca<sup>2+</sup>-dependent processes. Although the [Ca<sup>2+</sup>]<sub>c</sub> concentration peaks measured with Ca<sup>2+</sup> probes are usually underestimated, it is believed that highly localized and transient high [Ca<sup>2+</sup>]<sub>c</sub> microdomains (HCMD) may build up at specific locations (18, 86, 286, 347). In chromaffin cells, the first imaging experiment revealing localized [Ca<sup>2+</sup>]<sub>c</sub> elevations was done by O'Sullivan et al. (302). The likelihood for generation of a HCMD increases very much with the coincidence in time and space of the opening of several calcium channels, and this depends on four dimensions: the three spatial coordinates (*x*, *y*, *z*) and time (*t*). These four dimensions can be regarded as the degrees of freedom that oppose HCMD generation. In excitable cells, the *z* coordinate for the calcium channels is severely restricted, as the channel has to lie within the plasma membrane plane. In addition, channels may tend to pack together in clusters, thus introducing additional

restrictions on *x* and *y*. Finally, because the channel openings are synchronized by the action potential, the *t* dimension is also restricted. The result is that the probability for HCMD generation at the subplasmalemmal region and coincident in time with action potentials is very much increased in excitable cells.

The generation of HCMD by Ca<sup>2+</sup> release from the intracellular Ca<sup>2+</sup> stores may also be favored in certain circumstances. The spatial coordinates of the calcium channels of the stores are also restricted, as they can only locate at the ER membrane. In this case a specific HCMD topography can be favored by the geometric disposition of the ER or its spatial relation to other organelles or morphological differentiations, for example, mitochondria, nucleus, secretory vesicles, or dendritic spines (107, 108, 183, 330, 365, 370). Local gradients of channel agonists, such as InsP<sub>3</sub>, must also synchronize the opening of calcium channels that are physically close in space. CICR, which is observed for both ryanodine receptors and InsP<sub>3</sub> receptors (50), also tends to restrict the time span, thus increasing the probability of a HCMD.

Thus the Ca<sup>2+</sup> signaling system is designed to favor the generation of HCMD that are highly localized in space and time. This allows regulation of several different functions to be regulated by the same triggering signal, but at distinct subcellular locations and with different time patterns. Considerable attention has been given to the spatiotemporal profiles of the [Ca<sup>2+</sup>]<sub>c</sub> transients, which depend on Ca<sup>2+</sup> transport by the plasma membrane and cytosolic organelles, and Ca<sup>2+</sup> diffusion and buffering by the cytosol.

In chromaffin cells, cytosolic Ca<sup>2+</sup> buffering and diffusion have been extensively studied by Neher and co-

workers (414, 420). The cytosol has a  $\text{Ca}^{2+}$  binding capacity of  $\sim 4$  mmol/l cells. The endogenous  $\text{Ca}^{2+}$  buffer is scarcely mobile and has a low  $\text{Ca}^{2+}$  affinity ( $K_d \sim 100$   $\mu\text{M}$ ). The activity coefficient is  $\sim 1/40$  in bovine chromaffin cells (414, 420). The two-dimensional diffusion coefficient is  $\sim 40$   $\mu\text{m}^2/\text{s}$  and shows inhomogeneities at the nuclear envelope and at the plasma membrane (283). Brief openings of HVA calcium channels generate HCMD near the channel mouth, and then can be detected in  $\text{Ca}^{2+}$  imaging measurements (287). In these microdomains,  $\text{Ca}^{2+}$  can reach concentrations as high as 10  $\mu\text{M}$  and perhaps 100  $\mu\text{M}$  (35, 286). Because of rapid diffusion of  $\text{Ca}^{2+}$  towards the surrounding cytosol, the  $[\text{Ca}^{2+}]_c$  microdomains are very much restricted in time and space (285, 286). The presence of mobile  $\text{Ca}^{2+}$  buffers accelerates diffusion and opposes the development of the HCMD (16, 292, 336); at a concentration of 50  $\mu\text{M}$ , fura 2 increases the apparent rate of  $\text{Ca}^{2+}$  diffusion four times (420).

$\text{Ca}^{2+}$  entry through HVA calcium channels and its subsequent clearance have been extensively studied in bovine and rat chromaffin cells (for examples, see Refs. 194, 305, 414, 420). Membrane depolarization to 0 mV elicits  $\text{Ca}^{2+}$  currents peaking near 800 pA that deactivate with half-time constants of 300–500 ms. In bovine chromaffin cells, a depolarizing stimulus lasting for 0.5 s typically elicits a mean  $I_{\text{Ca}}$  of 250 pA. In terms of  $\text{Ca}^{2+}$  flow, for a 15- $\mu\text{m}$ -diameter cell, this current is equivalent to 700  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  (420). Measurements of  $^{45}\text{Ca}$  uptake by bovine chromaffin cells depolarized with high  $\text{K}^+$  (59 mM) are linear during the first 5 s at an estimated rate of  $0.7 \times 10^{-15}$  mol  $\cdot$  cell $^{-1} \cdot$  s $^{-1}$ , which is equivalent to 400  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  (29).

Plasma membrane  $\text{Ca}^{2+}$  extrusion is due to joint operation of both a plasma membrane  $\text{Ca}^{2+}$ -ATPase and a  $\text{Na}^+/\text{Ca}^{2+}$  exchange system. The joint action of both transport systems has been estimated to decrease  $[\text{Ca}^{2+}]_c$  to a maximal rate of  $\sim 0.2$   $\mu\text{M}/\text{s}$ , equivalent to 20  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$ , in rat chromaffin cells at 27°C (194, 305). At 37°C, the rate could be close to 100  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  (388).

Information on transport parameters by organelles in intact cells is usually indirect, and inferred from their effects on  $[\text{Ca}^{2+}]_c$ . Maximal uptake by ER has been reported to range between 1 and 1.5  $\mu\text{M}/\text{s}$  (equivalent to 40–60  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$ ), during stimulation of bovine (414) and rat (194) chromaffin cells. Direct measurements with ER-targeted aequorins give figures of up to 70–80  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  at 37°C (15, 388).  $\text{InsP}_3$ -producing agonists (bradykinin, histamine, angiotensin II, PACAP, muscarinic agonists, etc.), ATP, glutamate, and caffeine produce  $\text{Ca}^{2+}$  release from the ER (15, 21, 91, 94, 95, 97, 112, 113, 212, 213, 273, 297, 319, 338, 357–359). The rate of  $\text{Ca}^{2+}$  exchange at steady state is  $\sim 2$ –3  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  at rest, whereas the net leak induced by caffeine or by

maximal stimulation with  $\text{InsP}_3$ -producing agonists is 10–20 times faster (15). The ER behaves like a single homogeneous thapsigargin-sensitive pool from which  $\text{Ca}^{2+}$  can be released either via  $\text{InsP}_3$  or ryanodine receptors (15, 213). However, an earlier study showed distinct  $\text{InsP}_3$ - and caffeine-sensitive ER  $\text{Ca}^{2+}$  pools in bovine chromaffin cells (91). It must be remembered that  $\text{Ca}^{2+}$  entry through HVA channels activates release from ER in bovine chromaffin cells (see above). The amplitude of the CICR, quantified as ER  $[\text{Ca}^{2+}]$  decrease, depended on the luminal  $\text{Ca}^{2+}$  concentration and was facilitated by low concentrations of caffeine (15). In mouse chromaffin cells, the CICR is much smaller or nonexistent (326).

$\text{Ca}^{2+}$  transport by mitochondria has received renewed attention in the last few years, both because of possible participation in shaping  $[\text{Ca}^{2+}]_c$  transients and because changes of intramitochondrial  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_M$ ) seem important by themselves for the regulation of cell functions such as the respiration rate or programmed cell death (130, 328).  $\text{Ca}^{2+}$  is taken up through the  $\text{Ca}^{2+}$  uniporter, a low-affinity/high-capacity system (182). The driving force is the mitochondrial membrane potential,  $-150$  to  $-180$  mV, which would promote accumulation of  $\text{Ca}^{2+}$  into the mitochondrial matrix up to 5–6 orders of magnitude above  $[\text{Ca}^{2+}]_c$  at the thermodynamic equilibrium (49).  $\text{Ca}^{2+}$  exit from mitochondria takes place through a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and also through a  $\text{Na}^+$ -independent system, the former being dominant in the adrenal medulla (181, 182). The activity coefficient of  $\text{Ca}^{2+}$  inside the mitochondrial matrix seems to be very low, in the 1/1,000 range (38, 388). It has been shown that mitochondria can clear cytosolic  $\text{Ca}^{2+}$  loads in both rat (38, 194) and bovine (414) chromaffin cells. Herrington et al. (194) reported mitochondrial uptake rates of (in terms of changes of  $[\text{Ca}^{2+}]_c$ ) 0.4 to 0.7  $\mu\text{M}/\text{s}$  at  $[\text{Ca}^{2+}]_c$  concentrations of 0.5 to 2  $\mu\text{M}$ . Xu et al. (414) report much larger rates, 120  $\mu\text{M}/\text{s}$  (equivalent to 4,800  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$ ) at saturating  $[\text{Ca}^{2+}]_c$  concentrations (200  $\mu\text{M}$ ). These differences are consistent with the exponential  $[\text{Ca}^{2+}]_c$  dependence of the activity of the mitochondrial  $\text{Ca}^{2+}$  uniporter (269, 270). With the use of mitochondria-targeted aequorins, a value of  $\sim 160$   $\mu\text{M}/\text{s}$  (equivalent to 6,000  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$ ) for the maximal mitochondrial uptake has been reported (388). During maximal stimulation of  $\text{Ca}^{2+}$  entry by depolarization with high  $\text{K}^+$ , mitochondria take up  $\sim 1,100$   $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$  of  $\text{Ca}^{2+}$ .

The release of  $\text{Ca}^{2+}$  from  $\text{Ca}^{2+}$ -loaded mitochondria takes place mainly through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (181). The maximal rate at 37°C is  $\sim 20$   $\mu\text{M}/\text{s}$  (800  $\mu\text{mol} \cdot \text{l cells}^{-1} \cdot \text{s}^{-1}$ ) and the  $K_{50}$  is 200  $\mu\text{M}$  with exponential kinetics (388). The sensitivity of this system to temperature is very high. Transport through the uniporter is usually unidirectional, but when mitochondria are fully depolarized, the uniporter may mediate  $\text{Ca}^{2+}$  exit from the matrix. Under these circumstances, the increase of

$[Ca^{2+}]_c$  may trigger a paradoxical and massive release of  $Ca^{2+}$  from mitochondria (mitochondrial  $Ca^{2+}$ -induced  $Ca^{2+}$  release, mCICR; Ref. 269). Opening of the permeability transition pore can also produce mCICR (209).

The nuclear envelope may somewhat delay the propagation of  $Ca^{2+}$  waves from the cytosol to the nucleus (283). The half-equilibration times through the nuclear envelope are in the range of seconds (87). This may limit the progression of high-frequency  $[Ca^{2+}]_c$  oscillations to the nucleus. The nuclear matrix could also differ from the cytosol in having a larger  $Ca^{2+}$ -buffering power (368), and this would also result in an obvious slowing of the progression of the  $Ca^{2+}$  wave.

Secretory granules contain large amounts of calcium, but exchange through their membrane is too slow to contribute to  $[Ca^{2+}]_c$  transients (but see Ref. 309). However, there are several features that convert secretory vesicles into a unique candidate involved in  $Ca^{2+}$  homeostasis and in its participation in vesicle movements and exocytosis. Vesicles occupy nearly 20% of chromaffin cell volume. They contain  $\sim 40$  mM  $Ca^{2+}$ , although most of it is associated with vesicle matrix (mostly chromogranins);  $\sim 10\%$  of that is free inside the vesicles. Vesicle membranes are very rich in  $InsP_3$  receptors, which are supposedly directly linked to chromogranins (416). Also, the affinity of chromogranins for  $Ca^{2+}$  is strongly dependent on pH (417). Because the vesicular pH is modulated by second messengers like PKA and PKG (70) and the  $Ca^{2+}$  will be released as close as possible to its targets (see Table 2), it is likely that it will play a crucial role in all the vesicular processes dependent on  $Ca^{2+}$ . Recently, the intravesicular levels of free  $Ca^{2+}$  have been measured

using aequorin targeted to chromogranin A (254) as well as low  $Ca^{2+}$  affinity aequorin targeted to the inner fraction of the vesicular associated membrane protein (VAMP) (272). The study found  $Ca^{2+}$  basal levels of  $40 \mu M$  in PC12 cells; these granules release  $Ca^{2+}$  towards the cytosol as a response to depolarizing stimuli or caffeine; however, the authors did not observe dynamic changes of vesicular  $Ca^{2+}$  after application of  $InsP_3$ .

$Ca^{2+}$  homeostasis at each moment is defined by fluxes between three compartments: the extracellular medium, the cytosol, and the  $Ca^{2+}$ -storing organelles (Fig. 8). At rest, these fluxes are low, with exchange rates in the range of  $1\text{--}10 \mu mol \cdot l \text{ cells}^{-1} \cdot s^{-1}$  both at the plasma membrane and at the ER membrane. Mitochondrial  $Ca^{2+}$  transport through the uniporter is very slow because of the low  $Ca^{2+}$  affinity of this transport system and its exponential kinetics (A in Fig. 8B). The free  $Ca^{2+}$  concentrations at the steady state are in the range of  $10^{-7}$  M in the cytosol and the mitochondrial matrix and near  $10^{-3}$  M at the extracellular medium and inside the ER, and consequently, there are significant concentration gradients favoring  $Ca^{2+}$  diffusion to the cytosol.

When HVA calcium channels open during an action potential, inward  $Ca^{2+}$  current peaks of  $\sim 800$  pA (equivalent to  $2,000\text{--}3,000 \mu mol \text{ Ca}^{2+} \cdot l \text{ cells}^{-1} \cdot s^{-1}$ ) develop. At low stimulation, the rates of diffusion through the cytosol and binding by the endogenous  $Ca^{2+}$  buffers are the main determinants of the  $[Ca^{2+}]_c$  signal (285, 286).  $[Ca^{2+}]_c$  reaches  $10^{-6}$  M levels, and clearance through high-affinity  $Ca^{2+}$  pumps (plasma membranes and SERCA) dominates (B in Fig. 8B). At high stimulation rates,  $[Ca^{2+}]_c$  may reach levels high enough to stimulate

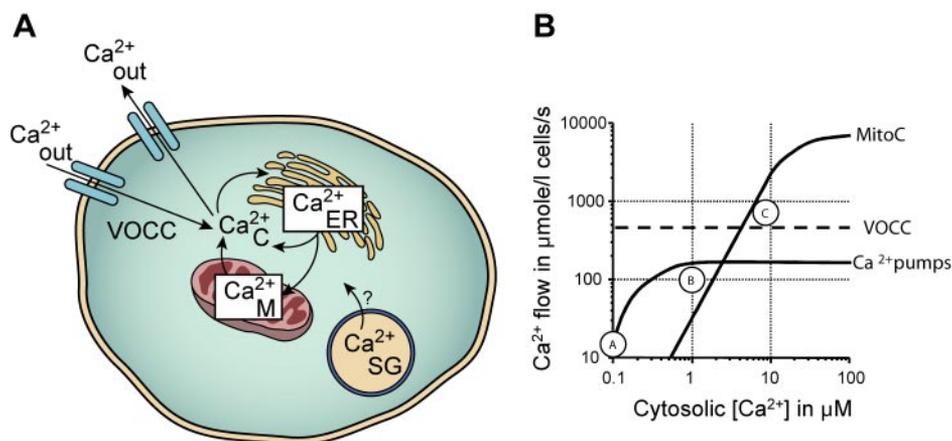


FIG. 8.  $Ca^{2+}$  homeostasis in adrenal chromaffin cells is defined by  $Ca^{2+}$  fluxes among different cellular and subcellular compartments. A:  $Ca^{2+}$  redistributes among different subcellular compartments in the transition from rest to activation, and vice versa. At rest,  $Ca^{2+}$  is stored at high concentrations in the endoplasmic reticulum (ER) and the secretory granules (SG) and at low concentrations in the cytosol (C) and mitochondria (M). During cell activation,  $Ca^{2+}$  enters the cell via plasma membrane calcium channels (VOCC) or is released from the ER, and the  $[Ca^{2+}]_c$  increases. Mitochondria (M) close to the release sites take up some of the released  $Ca^{2+}$ , and  $[Ca^{2+}]_M$  increases and stimulates respiration. Possible release of  $Ca^{2+}$  from SG (?) is a controversial issue. Once cell stimulation ceases,  $Ca^{2+}$ -ATPases in the ER and in the plasma membrane return  $[Ca^{2+}]_c$  to resting levels. [From Alvarez et al. (18).] B: concentration dependence of the different  $Ca^{2+}$  transport systems in bovine chromaffin cells. MitoC, mitochondrial uniporter;  $Ca^{2+}$  pumps, joint action of plasma membrane and SERCA ATPases. VOCC shows the rate of  $Ca^{2+}$  entry through plasma membrane calcium channels. A, B, and C represent rest, moderate, and strong stimulation, respectively. [From Villalobos et al. (387).]

transport through the  $\text{Ca}^{2+}$  uniporter (C in Fig. 8B). In this case, most of the  $\text{Ca}^{2+}$  load is taken up by mitochondria (194, 270, 305, 388, 414). It has been shown that, for sustained (10 s) depolarizations with a high- $\text{K}^+$  solution, >90% of the  $\text{Ca}^{2+}$  load is taken up by mitochondria during the stimulation period. Once stimulation ceases, mitochondrial  $\text{Ca}^{2+}$  is released to the cytosol during a period of seconds or minutes (388).  $[\text{Ca}^{2+}]_c$  remains discretely elevated during this period, and this may help to mobilize secretory vesicles from the reserve pool toward the membrane, where they will be ready for use in the next exocytotic episode (286).  $\text{Ca}^{2+}$  accumulated in mitochondria stimulates respiration (181, 328). This may help to provide extra energy for clearing the  $\text{Ca}^{2+}$  load up to quickly restore cell homeostasis after the activity period.

Pioneering work from the laboratories of Chad and Eckert (86) and Simon and Llinás (347) provided the first modeling of the HCMD expected to evolve around an open channel, which were later evidenced experimentally (241). In chromaffin cells, opening of calcium channels generates microdomains of 0.3  $\mu\text{m}$  diameter at 5–10  $\mu\text{M}$   $[\text{Ca}^{2+}]_c$  (99, 228, 287, 395). Clusters of HVA calcium channels and synaptic vesicles (325) colocalize in chromaffin cells (300, 332, 419). Recent simultaneous measurements of  $[\text{Ca}^{2+}]_c$  and exocytosis at submicrometer resolution with evanescent microscopy demonstrated fast ( $t_{1/2}$ , 100 ms) and localized (350 nm diameter) hot spots of high  $\text{Ca}^{2+}$  (47). Only the docked vesicles that are located 300 nm away from a site of  $\text{Ca}^{2+}$  entry could be released quickly and only ~10% of these vesicles (the “primed” ones) were exocytosed (47, 300).

Since HCMD generate near HVA calcium channels, mitochondrial  $\text{Ca}^{2+}$  uptake could occur locally at these places during physiological stimulation (194, 305, 388). Single cell measurements in anterior pituitary cells, which have spontaneous electric and  $[\text{Ca}^{2+}]_c$  activity, showed

spontaneous oscillations of  $[\text{Ca}^{2+}]_M$  (387). Measurements in chromaffin cells have demonstrated that  $\text{Ca}^{2+}$  entering through HVA calcium channels is taken up by a pool of mitochondria close to the plasma membrane (270, 388). This uptake stops the progression of the  $\text{Ca}^{2+}$  wave towards the cell core (Fig. 9A). The two mitochondrial pools of bovine adrenal chromaffin cells (M1 and M2) take up  $\text{Ca}^{2+}$  at very different rates (in  $[\text{Ca}^{2+}]_c$  units), >50  $\mu\text{M/s}$  and 0.3  $\mu\text{M/s}$ , equivalent to 2,000 and 12  $\mu\text{mol} \cdot \text{cells}^{-1} \cdot \text{s}^{-1}$ , respectively (270, 388). These rates are reached at ~20 and 2  $\mu\text{M}$   $[\text{Ca}^{2+}]_c$ , which would correspond to the  $\text{Ca}^{2+}$  concentrations reached at the subplasmalemmal region, where exocytosis takes place, and at the cell core, respectively (Fig. 9B). The increase of  $[\text{Ca}^{2+}]_M$  in pool M1 would keep respiration increased and provide the basis for subcellular tuning of the mitochondrial function to match the local energy needs (ATP in Fig. 9B) as well as perhaps other factors required for the secretory process (411). The remaining mitochondria, at the bulk cytosol, would sense a much smaller  $[\text{Ca}^{2+}]_c$  increase. A similar compartmentalization of  $\text{Ca}^{2+}$  signaling by mitochondrial function has been suggested in frog sympathetic neurons (314) and pancreatic acinar cells (304, 373).

The CICR may amplify the  $\text{Ca}^{2+}$  signals generated by  $\text{Ca}^{2+}$  entry through VOCC. Measurements of ER  $[\text{Ca}^{2+}]$  during stimulation with high  $\text{K}^+$  show net decreases of 60–100  $\mu\text{M}$  (10–15% of the total content) with each stimulating pulse (15). This may seem quite a small amount of  $\text{Ca}^{2+}$ , but its averaged value may be composed of strong liberation in certain places largely compensated by strong uptake in others. As a matter of fact, stimulation with high  $\text{K}^+$  does produce an increase of ER  $[\text{Ca}^{2+}]$  in other cell types that do not possess CICR (15). In addition, the strength of CICR may be regulated. Although we were unable to find stimulation by cADP ribose, we found that

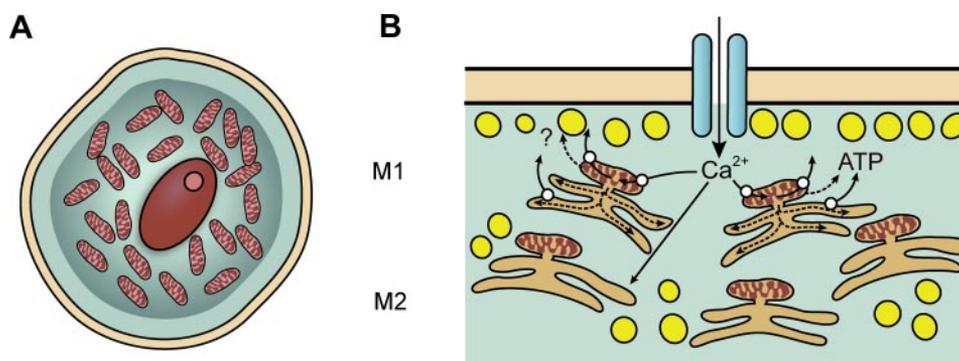


FIG. 9. Effect of mitochondrial  $\text{Ca}^{2+}$  transport on the progress of the  $\text{Ca}^{2+}$  wave through the cytosol. A: the high  $\text{Ca}^{2+}$  transient (dots) is confined to the subplasmalemmal region because most of the  $\text{Ca}^{2+}$  diffusing towards the cell core is taken up by mitochondria beneath the plasma membrane (gray, to indicate that they become loaded with  $\text{Ca}^{2+}$ ). The core cytosol and mitochondria in this region contain much smaller  $[\text{Ca}^{2+}]$ . B: the high  $\text{Ca}^{2+}$  microdomain is restricted to the subplasmalemmal region close to the open channel and affects only some of the secretory vesicles.  $\text{Ca}^{2+}$  is taken up through the mitochondrial  $\text{Ca}^{2+}$  uniporter and stimulates respiration in the  $\text{Ca}^{2+}$ -loaded mitochondria, thereby increasing local ATP synthesis and perhaps other intermediates required for exocytosis (388). Pools of mitochondria close (M1) and far away (M2) from the plasma membrane are shown. There could be some degree of continuity of the mitochondrial space among different mitochondria.

the CICR was sensitized by low concentrations of caffeine or by raising the  $\text{Ca}^{2+}$  stored inside the ER. On the other hand, fast confocal measurements showed that the  $[\text{Ca}^{2+}]_c$  wave induced by 100-ms depolarizing pulses was delayed and reduced in ryanodine-treated cells (15). CICR seems to colocalize with plasma membrane calcium channels and the mitochondrial pool that undergoes large  $[\text{Ca}^{2+}]_M$  changes during depolarization (Fig. 10). Thus complex functional units, gathering all the elements needed to control the subplasmalemmal  $[\text{Ca}^{2+}]_c$  transients near the exocytotic sites together, seem to exist in chromaffin cells (270).

We believe that functional triads that include the HVA calcium channels, the mitochondrial  $\text{Ca}^{2+}$  uniporter, and the ryanodine receptor from the ER are responsible for the generation of local high  $[\text{Ca}^{2+}]_c$  transients that control the rate and extent of catecholamine release. The local  $[\text{Ca}^{2+}]_c$  hot spots may reach concentrations of  $\sim 50 \mu\text{M}$  at the subplasmalemmal region where the secretory

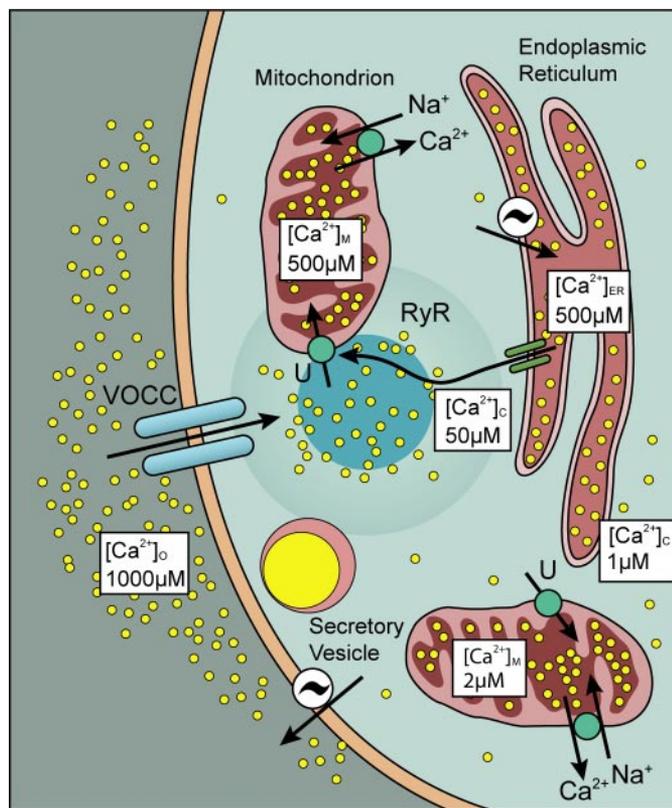


FIG. 10. Functional triads responsible for the generation of local high  $\text{Ca}^{2+}$  transients and catecholamine secretion in chromaffin cells. The voltage-dependent calcium channel (VOCC), the  $\text{Ca}^{2+}$  uniporter (U) from the surrounding mitochondria, the ryanodine receptor (RyR) from the endoplasmic reticulum, and the secretory vesicle are all strategically located beneath the plasma membrane.  $\text{Ca}^{2+}$  entry through the VOCC triggers  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the RyR and generates a local  $[\text{Ca}^{2+}]_c$  microdomain of  $\sim 50 \mu\text{M}$ . In the rest of the cell, the  $[\text{Ca}^{2+}]_c$  increase was much smaller,  $\sim 1 \mu\text{M}$ . The mitochondrion, located far away from the high  $\text{Ca}^{2+}$  microdomain, increases its  $[\text{Ca}^{2+}]_M$  only to  $\sim 2 \mu\text{M}$ . [From Montero et al. (270).]

vesicles are docked. The calcium channel acts as the trigger, the ryanodine receptor located at strategic places, as the signal amplifier, and the mitochondria as a contention wall that avoids propagation of the high  $\text{Ca}^{2+}$  tide to the cell core, where such a huge signal is not required. In addition,  $\text{Ca}^{2+}$  taken up by mitochondria stimulates respiration, thus tuning up energy production to support the increased requirements from the exocytotic activity. Respiratory stimulation will lag behind the cessation of activity until the mitochondrial  $\text{Ca}^{2+}$  load is completely cleared. Much of the  $\text{Ca}^{2+}$  that enters the mitochondria at subplasmalemmal locations may diffuse through the mitochondrial matrix to other cell locations and eventually be extruded from mitochondria near the cell core. This mitochondrial  $\text{Ca}^{2+}$  release probably contributes to keeping  $[\text{Ca}^{2+}]_c$  discretely raised during the poststimulus period, perhaps facilitating the transport of new vesicles to refill the RRP (396).

We can speculate that if either the location or the  $\text{Ca}^{2+}$  uptake properties of the mitochondria in these functional triads could be modulated, this would be an effective mechanism to regulate the exocytotic process. If similar phenomena could be extrapolated to neurons, this would also be a simple mechanism to modulate synaptic plasticity. Under pathological conditions (i.e., excitatory neurotoxicity, ischemia-reperfusion) or during ageing or Alzheimer's, Parkinson's, or other neurodegenerative diseases, mitochondrial damage may reduce the ability of the mitochondria to take up  $\text{Ca}^{2+}$ . This would lead to increased secretion of excitatory neurotransmitters and increased cell activation, a vicious circle that may trigger processes leading to necrosis or apoptosis. Changes in CICR would also modulate the synaptic efficacy under physiological or pathophysiological conditions. A capacitative  $\text{Ca}^{2+}$  entry pathway activated by ER  $\text{Ca}^{2+}$  depletion has also been described in chromaffin cells (96); this pathway has been implicated in the regulation of exocytosis (150).

## VIII. CONCLUSIONS AND PERSPECTIVES

Since the pioneering work of Douglas and Rubin in 1961 (126) showing that  $\text{Ca}^{2+}$  entry was the only requirement to trigger the acetylcholine-mediated release of catecholamines from the perfused cat adrenal gland, we have learned much about basic and molecular aspects of  $\text{Ca}^{2+}$  signaling and exocytosis in chromaffin cells. However, this large body of knowledge has generated many new questions that will be explored during the next decade in detail. The combination of powerful imaging techniques (i.e., evanescent wave microscopy) with amperometry and patch-clamp associated with capacitance measurements, patch amperometry, genetic manipulation (transgenic animals), molecular biology, and labeling of secre-

tory machinery proteins, as well as new specific neurotoxins and pharmacological tools, will generate continuous new knowledge, answering these questions and raising new questions.

In 20 years of  $\omega$ -toxin use, at least six subtypes of calcium channels have been identified and characterized. New toxins are needed to selectively target the P/Q-type calcium channel without affecting the N channel. The R- or T-type channels also need new toxins to characterize their functions. Whether the P and Q channels are the same or separate entities in various cell types remains to be clarified. The question of how many calcium channel subtypes remain to be discovered is also relevant. In addition, differences between tissues and cell types for a given calcium channel are emerging; L-type calcium channels are different in skeletal, cardiac, smooth muscle, and brain. Are the P/Q channels from hippocampal and chromaffin cells identical? What about the N, P, or R channels? Why are different calcium channels required to control exocytosis of the same transmitter (i.e., acetylcholine, catecholamines) in the same cell type in different animal species? Another important question relates to the expression of various channel subtypes in the same cell. Why does exocytosis require  $\text{Ca}^{2+}$  from different pathways? Is this a safety valve to ensure the efficiency of the process? If the N channel is a part of the secretory machinery, what about the L or P/Q channels? How close are they to the exocytotically active sites? And most interesting, are the channels of a paraneuronal cell such as the chromaffin cell organized the same as those brain synapses? Why is the release of norepinephrine controlled by N channels in sympathetic neurons and by L or P/Q channels in chromaffin cells? Do action potentials recruit different calcium channel subtypes in these two catecholaminergic cell types? Will  $\text{K}^+$  or square depolarizing stimuli recruit calcium channels different from those recruited by action potentials in neurons, or by acetylcholine receptors in chromaffin cells? Is the electrical pattern of different excitable cells producing different secretion patterns by simply recruiting specific calcium channels with particular gating and kinetic properties? It will be interesting to explore how the chromaffin cell organelles contribute to shaping the  $[\text{Ca}^{2+}]_c$  elevations and the exocytotic responses generated by different stimulation patterns and calcium channel subtypes. Again, it will be highly relevant to explore all these questions *in situ* in fresh adrenal slices and, if possible, in organotypic cultures from such slices.

The idea that some subtype of calcium channel might colocalize more tightly with certain SNARE proteins is emerging. Also, it is plausible that vesicles dock at restricted areas of the plasma membrane sites, where docking proteins accumulate. This colocalization is unclear in cultured chromaffin cells; however, as techniques to explore  $\text{Ca}^{2+}$  signals in cell compartments and exocytosis

are applied to cells in adrenal slices *in situ*, new, more physiological aspects of this possible colocalization will emerge. The chromaffin cell is polarized *in situ*; this polarization and the expression of calcium channels and other proteins are altered in primary cultures. Hence, adrenal slice techniques must be potentiated, since they may provide new clues to the stimulus-secretion coupling process in more physiological conditions.

We need to understand the molecular basis of priming, the process by which vesicles acquire fusion competence. In addition, the early steps of exocytosis need to be explored further to somewhat know which molecules and mechanisms are responsible for the recruitment and guidance of vesicles to their docking sites. Several molecules might be involved, for instance, clusters of SNARE proteins serving as docking sites (232), Munc18-1 as a docking factor (325), calcium channel synprint domains involved in the interaction between L-type calcium channels and SNARE proteins (216), Munc13-1, 25-kDa synaptosomal-associated proteins (SNAP-25), complexin and protein kinase C as priming factors (325, 352, 374), and cytoskeletal proteins such as molecular motors and rails for vesicle transport (296).

The chromaffin cell has been an ideal unique model to study basic mechanisms of  $\text{Ca}^{2+}$  signals and exocytosis; these studies have often been the basis to understand issues related to neuronal communication. We predict that this cell model will continue to be invaluable in exploring these mechanisms with new high-resolution techniques.

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We apologize to those authors whose work related to calcium signaling and exocytosis in chromaffin cells has not been cited in this review; space restrictions required a selection of topics in this vast field.

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