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INVITED REVIEW

# L-type calcium channels in exocytosis and endocytosis of chromaffin cells

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**Abstract** The coexistence of different subtypes of voltage-dependent calcium channels (VDCC) within the same chromaffin cell (CC) and the marked interspecies variability in the proportion of VDCC subtypes that are present in the plasmalemma of the CCs raises the question on their roles in controlling different physiological functions. Particularly relevant seems to be the role of VDCCs in the regulation of the exocytotic neurotransmitter release process, and its tightly coupled membrane retrieval (endocytosis) process since both are $Ca^{2+}$-dependent processes. This review is focused on the role of $Ca^{2+}$ influx through L-type VDCC in the regulation of these two processes. It is currently accepted that the different VDCC subtypes (i.e., T, L, N, P/Q, R) contribute to exocytosis proportionally to their density of expression and gating properties. However, the pattern of stimulation defines a preferential role of the different subtypes of VDCC on exocytosis and endocytosis. Thus, L-type channels seem to control catecholamine release induced by prolonged stimuli while fast exocytosis in response to short square depolarizing pulses or action potentials is mediated by $Ca^{2+}$ entering CCs through P/Q channels. The pattern of stimulation also influences the endocytotic process, and thus, electrophysiological data suggest the sustained $Ca^{2+}$ entry through slow-inactivating L-type channels could be responsible for the activation of fast endocytosis.

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## Introduction

The "fight or flight" response constitutes a highly coordinated and precise response physiologically generated as an attempt for maintaining the equilibrium of the internal milieu against fear or stress conflicts [15, 19]. This response is highly regulated by the sympathetic nervous system, being particularly relevant the participation of the chromaffin cells (CCs) of the adrenal gland that release the catecholamines adrenaline and noradrenaline, a response that is dependent on extracellular $Ca^{2+}$ [33] that enters the CC upon opening of different voltage-dependent $Ca^{2+}$ channels (VDCCs) present in their plasma membrane [43].

As it happens for other neurotransmitters and hormones, the $Ca^{2+}$-dependent release of catecholamines is highly dependent on the preservation of the equilibrium between the amount of vesicular membrane that incorporates into the plasmalemma during the exocytotic process and the membrane retrieval during subsequent endocytosis. This will serve to warrant that a given number of secretory vesicles are available to participate in subsequent rounds of exocytosis during repetitive cell activation [10, 27, 50]. Both exocytosis and endocytosis processes are mediated by a rise in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) achieved primarily by $Ca^{2+}$ entry through VDCCs [16, 27, 74, 84].

The identification and characterization of the properties, the regulation, and the functional role of the different subtypes of VDCC have been possible thanks to the improvement of the patch-clamp techniques [49], the isolation, the purification and synthesis of different neurotoxins [76], and the molecular biology and genetic approaches that have led to the

elucidation of the molecular structure of VDCCs [26]. The main properties of the different subtypes of VDCC, including the major pore-forming subunit and their pharmacological profile are summarized in Table 1.

By combining electrophysiological techniques and selective blockers of VDCC, we have found that the whole-cell inward $I_{Ca}$ of bovine chromaffin cells (BCCs) is mainly mediated by $Ca^{2+}$ entry through, at least, three of the subtypes of VDCCs described in neurons [76], namely, 20% L-type ($\alpha_{1D}$, Cav 1.3), 30% N-type ($\alpha_{1B}$, Cav 2.2), and 50% P/Q-type ($\alpha_{1A}$, Cav 2.1) [4, 5, 37, 40, 80]. The coexistence of these three subtypes of VDCCs within the same CC raises the question on their roles in controlling different physiological functions, particularly the implication of each VDCC subtype in

**Table 1** Voltage-dependent calcium channel subtypes (adapted from [7, 26, 43])

| Channel type | Pore-forming subunit | Type of current | Blockers | Activators | Tissue location | Function |
|---|---|---|---|---|---|---|
| Cav 1.1 | $\alpha_{1S}$ | L | Nifedipine<br>Calcicludine<br>Calciseptine<br>Diltiazem<br>Verapamil | BAY-K-8644<br>FPL64176 | Skeletal muscle | Excitation-contraction coupling |
| Cav 1.2 | $\alpha_{1C}$ | L | Nifedipine<br>Calcicludine<br>Calciseptine<br>Diltiazem<br>Verapamil | BAY-K-8644<br>FPL64176<br>PCA5094 | Heart<br>Smooth muscle<br>Brain<br>Pituitary<br>Endocrine cells<br>Adrenal medulla | Excitation-contraction coupling<br>Hormone release<br>Regulation of transcription<br>Synaptic integration |
| Cav 1.3 | $\alpha_{1D}$ | L | Verapamil<br>Less sensitive to DHP antagonists | BAY-K-8644<br>FPL64176<br>PCA50941 | Brain<br>Pancreas<br>Adrenal medulla<br>Cochlea<br>Kidney<br>Ovary | Hormone release<br>Regulation of<br>Transcription<br>Synaptic regulation<br>Cardiac pacemaking<br>Repetitive firing<br>Hearing<br>Neurotransmitter release from sensory cells |
| Cav 1.4 | $\alpha_{1F}$ | L | Less sensitive to DHP antagonists | BAY-K-8644<br>FPL64176 | Retina | Neurotransmitter release from photoreceptors |
| Cav 2.1 | $\alpha_{1A}$ | P/Q | ω-aga- IVA<br>ω-ctx-MVIIC<br>ω-ctx-MVIID | | Cerebellum<br>Pituitary<br>Cochlea<br>Adrenal medulla | Neurotransmitter release<br>Dendritic $Ca^{2+}$ transients<br>Hormone release |
| Cav 2.2 | $\alpha_{1B}$ | N | ω-ctx-GVIA<br>ω-ctx-MVIIA<br>ω-ctx-MVIIC | | Brain<br>Peripheral nervous system<br>Adrenal medulla | Neurotransmitter release<br>Dendritic $Ca^{2+}$ transients<br>Hormone release |
| Cav 2.3 | $\alpha_{1E}$ | R | SNX-482 | | Brain<br>Cochlea<br>Retina<br>Heart<br>Pituitary<br>Adrenal medulla | Repetitive firing<br>Dendritic $Ca^{2+}$ transients |
| Cav 3.1 | $\alpha_{1G}$ | T | Mibefradil<br>Kurtoxin<br>Low sensitivity to $Ni^{2+}$ | | Brain<br>Peripheral nervous system<br>Adrenal medulla | Pacemaking; repetitive firing |
| Cav 3.2 | $\alpha_{1H}$ | T | Mibefradil<br>Kurtoxin<br>High sensitivity to $Ni^{2+}$ | | Heart<br>Brain<br>Kidney<br>Liver | Pacemaking; repetitive firing |
| Cav 3.3 | $\alpha_{1I}$ | T | Mibefradil<br>Kurtoxin<br>Low sensitivity to $Ni^{2+}$ | | Brain | Pacemaking; repetitive firing |

*DHP* dihydropyridines, *ω-aga-IVA* ω-agatoxin IVA, *ω-ctx-GVIA* ω-conotoxin GVIA, *ω-ctx-MVIIA* ω-conotoxin MVIIA, *ω-ctx-MVIIC* ω-conotoxin MVIIC, *ω-ctx-MVIID*, ω-conotoxin MVIID

the regulation of the two main $Ca^{2+}$-dependent steps involved in the neurotransmitter release process, i.e., the exocytotic release of catecholamines and the subsequent endocytotic process [43, 66]. In this review, we will focus on the $Ca^{2+}$ influx into the chromaffin cell through L-type VDCC that serves to regulate both the exocytosis and the endocytosis processes. In addition, growing evidence suggest that L-type (Cav1.2 and Cav1.3) channels are also directly involved in the repetitive firing of spontaneous [69, 71, 72] and evoked AP firings [89, 90].

## L-type $Ca^{2+}$ channels in chromaffin cells

The presence of L-type currents has been electrophysiologically characterized in bovine [4, 11, 12, 17, 18, 20, 37], rat [6, 28, 32, 35, 38, 69, 78], mouse [52, 67, 71, 72], pig [58], cat [2, 62], and human CCs [42, 55].

A comparative study has shown a high interspecies variability in the proportion of L-type VDCCs that are present in the plasmalemma of the CCs. Thus, L-type calcium channels account for near half of the whole-cell $Ca^{2+}$ channel current in the cat [2], rat [38], and mouse CCs [52], while in pig [58], bovine [4, 37], and human species [42] L channels carry only 15–20% of the whole-cell $Ca^{2+}$ current measured at holding voltage of about −70 to −80 mV. In addition, within the same animal species, age-dependent differences have also been described, i.e., in rat embryo CCs (RECCs) whole-cell $I_{Ca}$ is carried 60% by L channels in comparison with 50% found in adult rat CCs [36].

At this point, it should be mentioned that the estimate of L-type channels expression based on the action of dihydropyridines (DHPs) is highly sensitive to the holding potential [67]. Thus, for instance, in a recent study conducted in human CCs, the block of $Ca^{2+}$ currents by nifedipine at −80 mV is 20%, but increases to 50% at −50 mV [55]. These differences could be partially related to the voltage-dependent inactivation of non L-type VDCCs as will be discussed below.

Molecular evidence indicates that L-type currents in CCs is mediated by the expression of two subtypes of L channels, $\alpha_{1C}$ and $\alpha_{1D}$ [13, 46, 47, 55, 66, 92], and the most common view is that CCs express equal percentages of Cav1.2 and Cav1.3 L-type channels [68, 72]. However, on the basis of their affinities for DHPs, from RT-PCR and from single-channel recordings, it is difficult to separate the contribution of these two channel types to the total L-type current [67, 72, 88]. Also, using Cav1.3 KO mice show clearly that both isoforms are equally modulated by cAMP and cGMP [68].

At this point, we would like to comment that, in order to characterize the functional role of L-type VDCC, some characteristics that differentiate L-type channels from other VDCCs should be considered, as these could contribute to explain some of the discrepancies observed between different studies. These differences are related to (1) the different autocrine/paracrine regulation by catecholamines and other co-exocytosed vesicular components (the L current is regulated by neurotransmitters in a voltage-independent manner while N and PQ currents are regulated in a voltage-dependent manner [3, 20, 39, 51]), (2) the voltage-dependent inactivation (N and PQ channels undergo a pronounced voltage-dependent inactivation while L channels are resistant to such inactivation [53, 91]), and/or (3) the $Ca^{2+}$-dependent inactivation (L-type channels undergo $Ca^{2+}$-dependent inhibition at a rate slower than that of N and PQ-type channels [54, 80]). Finally, it should be noted that the number of $Ca^{2+}$-channel and its distribution might be also altered by culturing conditions as result of denervation/isolation of the CCs.

## L channels and exocytosis in chromaffin cells

Some discrepancies on the role of the different subtypes of VDCCs on the regulation of the exocytotic process have been published. These differences are somehow related to the different stimulation patterns used (i.e., stimulation with the physiological neurotransmitter acetylcholine, $K^+$ depolarization, electrical stimulation, short or long stimulation, …), the preparation used (i.e., intact gland, adrenal slices, cultured cell populations, or cultured isolated cells), and/or the techniques used to quantify the catecholamine secretion (i.e., amperometry in cell populations or in single cell, cell capacitance in patch-clamped cells, …).

For instance, in the intact adrenal gland of the cat, the $K^+$-evoked secretion of catecholamines is effectively blocked in a concentration dependent manner by DHPs and by other drugs acting on L-type VDCCs like verapamil and diltiazem [25, 41] and markedly potentiated by the DHP agonist BAY-K-8644 [44] thus suggesting that catecholamine secretion in these cells was mainly controlled by an L-type channel. However, electrophysiological experiments demonstrated that cat CCs also contained N-type channels in a similar proportion to that of L-type channels [2]. Further experiments showed that though $Ca^{2+}$ entry through both channels (N- and L-type) lead to similar increments of the average $[Ca^{2+}]_c$, the control of $K^+$-evoked catecholamine release response in cat chromaffin cells was dominated by $Ca^{2+}$ entering through L-type VDCCs [62].

In the intact rat adrenal gland, it was reported that the L-type VDCC blocker isradipine partially inhibited electrical stimulation- and acetylcholine-induced catecholamine secretion, but potently inhibited nicotine- and $K^+$-induced secretion in the perfused rat adrenal gland. In addition, BAY-K-8644 potentiated mildly the secretory responses to electrical stimulation and to acetylcholine, but increased threefold the responses to $K^+$ and nicotine. These results suggested that responses mediated by high $K^+$ or nicotinic receptors are mediated by $Ca^{2+}$ entry through L-type channels, although other VDCCs also

contributes to modulate the physiological adrenal catecholamine secretory process [63].

In a similar study, the catecholamine release induced by electrical field stimulation of splanchnic nerves was halved either by ω-conotoxin MVIIC (a non L-type channel blocker) and the DHP furnidipine, thus suggesting that both the L- and P/Q-types of $Ca^{2+}$ channels were involved. Similar results were observed when secretion was elicited by acetylcholine. However, the $K^{+}$-induced secretory responses were reduced 75% by furnidipine and 45% by ω-conotoxin MVIIC, indicating that this type of stimulation preferentially recruited L-type channels [82]. Similarly, Nagayama et al. found that L-type channels were responsible for the catecholamine secretion mediated by nicotinic receptors but not by muscarinic receptors, and that their contribution to noradrenaline secretion may be greater than that of adrenaline secretion. N-type voltage-dependent $Ca^{2+}$ channels may not contribute to catecholamine secretion, and P/Q-type $Ca^{2+}$ channels may control the secretion at presynaptic sites [73].

By using bovine chromaffin cell populations stimulated with $K^{+}$ depolarization, it was first concluded that $Ca^{2+}$ entry through both L- and P/Q-type channels controlled the $K^{+}$-evoked catecholamine release responses [64], in spite that L-type channels account for only 20% of the whole-cell currents in these cells. These results led to the hypothesis that L and P/Q channels were strategically located close to the secretory machinery, thus regulating the exocytosis of catecholamines [59, 64]. In a similar study conducted in distinct populations of bovine chromaffin cells, it was described that exocytosis in noradrenaline-containing cells was regulated mainly by L-type channels, while in adrenaline-containing cells exocytosis was controlled by P/Q-type channels [61].

However, when the possible coupling between VDCCs and exocytosis was evaluated at the single-cell level by measuring membrane capacitance, no preferential role of any VDCC subtype in eliciting exocytosis has been found in rat [48, 57] or in bovine CCs [34, 85, 83, 65, 87], thus suggesting an uneven distribution of calcium channels in chromaffin cells. A possible explanation for these discrepancies could be, at least partially, related to the voltage-dependent inactivation of VDCCs that minimizes the role of N and PQ channels in the experiments conducted in intact adrenal glands or in isolated cell populations, in which the physiological resting membrane potential of the chromaffin cells might favor a partial voltage-dependent inactivation of non L channels, while L channels are more resistant to such type of inactivation [53, 91].

Some striking differences have been observed related to the role of the different VDCC subtypes in the regulation of hypoxia-induced catecholamine secretion (HIS response). Thus, during fetal and neonatal periods in which there is no functional innervation of the adrenal medulla, a non-neurogenic acute HIS response is produced that depends on $Ca^{2+}$entry through VDCCs of CCs, as is proven by the fact that this response is abolished in the absence of $Ca^{2+}$ [1] and blocked by cadmium [45]. Different studies have concluded that this acute HIS response is mainly controlled by L channels in fetal sheep CCs [1], embryonic rat CCs [36], and neonatal rat CCs [85, 86]; However, the study by Levitsky and López-Barneo suggests that neonatal rat CCs express relatively high levels of T-type VDCCs and that the function of these channels is required for a proper secretory response to acute hypoxia [60]. On the other hand, by using both electrophysiological and molecular biology tools, it has been demonstrated that chronic hypoxia up-regulates the expression of T-type channels in adult CCs [22, 23, 70, 83]. These data are in good agreement with the idea that hypoxia, like other stress-mimicking conditions, up-regulates T-type channels in CCs [56, 75].

Finally, it has been proposed that channel gating and the type of stimuli applied, rather than the possible co-localization of the exocytotic machinery with VDCCs, regulate the exocytosis in chromaffin cells. Thus, as commented above, the different experimental approaches used during the last 30 years, mostly based on the application of a long-lasting stimulus, i.e., prolonged stimulation with high $K^{+}$ containing solutions [62] or acetylcholine [73], support the idea of a preferential coupling of L-type VDCCs to catecholamine secretion. However, a predominant role of P/Q-type channels in regulating the fast release of vesicles from the immediately releasable pool (IRP) has been proposed when short (10 ms) stimulation with square depolarizing pulses [8, 9] or trains of action potentials [29] are used to stimulate catecholamine secretion in mouse CCs. This seems to be likely due to the rapid activation of P/Q channels (Cav2.1) with respect to the other VDCCs which is more evident during stimuli of short duration since less affected by fast channel inactivation. The slow-inactivating L-type channels would be regulating the vesicular replenishment of the releasable pool, that is, the sustained or tonic release [24].

## L channels and endocytosis in chromaffin cells

As commented above, the $Ca^{2+}$-dependent release of catecholamines is highly dependent on the preservation of the membrane equilibrium between the amount of vesicular membrane that incorporates into the plasmalemma during the exocytotic process and the membrane retrieval during subsequent compensatory endocytosis.

In trying to characterize the possible relationship between $Ca^{2+}$ entry, exocytosis, and endocytosis by measuring changes in membrane capacitance ($\Delta$Cm) in BCCs, we found that $Ca^{2+}$ entry through VDCCs induced by the application of depolarizing pulses (DPs) of increasing length (50–2000 ms) produced different patterns of exo/endocytosis. A linear relationship between exocytotic responses and DP duration was found; however, endocytotic responses were almost absent

when short DPs (50–200 ms) were applied and were more pronounced with longer DPs (500–2000 ms) [31]. These data pose the question on whether the same $Ca^{2+}$ entry that triggers exocytosis is also responsible to initiate subsequent endocytosis.

As far as the specific contributions of the different VDCC subtypes in controlling endocytosis are concerned, it has been proposed that, as for exocytosis, the pattern of stimulation, and therefore, the characteristics of the $Ca^{2+}$ signal generated by the stimulus also influence endocytosis [24].

In bovine CCs stimulated with single DPs of long (500 ms) duration, a preferential coupling of L-type VDCCs to endocytosis has been proposed [79]. In this study, we found that, despite the small contribution of L-type VDCCs to the total global $Ca^{2+}$ current, their inhibition by the DHP nifedipine almost completely abolished the endocytotic response without significantly affecting exocytosis. ω-Conotoxin GVIA (N-channel blocker) affected little the exo/endocytotic responses while ω-agatoxin IVA (P/Q-channel blocker) markedly blocked those responses in a parallel manner. These data support the hypotheses that $Ca^{2+}$-entry through L channels is more effective in triggering endocytosis than exocytosis [79]. Additional experiments were performed with the isolation of L from N/PQ channels by blocking the non L channels with ω-conotoxin MVIIC (MVIIC). It was found that, in cells treated with MVIIC, superfusion with FPL64176 (an L-type VDCC agonist) increased $Ca^{2+}$ entry and doubled the endo/exocytosis ratio, indicating a selective augmentation of endocytosis related to this $Ca^{2+}$ entry through L-type channels [81]. Similar results were obtained by using the FM-dye methodology and long stimulations with high $K^+$; endocytosis was inhibited by about 50% when the L-channel blocker nifedipine was present [81].

Bay et al. (2012) have also reported the implication of L-type VDCC in the membrane excess retrieval that follows a strong $Ca^{2+}$ entry in mouse CCs. In this study, excess retrieval (a rapid endocytosis process that retrieves more membrane than the one fused by preceding exocytosis) was monitored with FM1.43 after the stimulation with high-$K^+$ or cholinergic agonists lasting for 15–30 s. It was found that this excess retrieval membrane pool is associated with the generation of a non-releasable fraction of membrane co-localizing with the lysosomal compartment and is controlled by the concerted contribution of extracellular and intracellular $Ca^{2+}$ sources. The blocking of the L-type VDCC with nitrendipine suppressed excess retrieval [14].

In trying to characterize if this preferential role of L-type VDCCs in controlling endocytosis was related to the existence of a close co-localization between endocytosis proteins, such as dynamin and/or clathrin, and L-type channels, we performed immunofluorescence experiments on bovine CCs that showed a practically negligible co-localization of clathrin with the three VDCC subtypes ($Ca_V1.3$, $Ca_V2.1$, and $Ca_V2.2$) studied. Also, only a mild co-localization (about 20–30%) was observed between VDCCs and dynamin. Taken together, these experiments do not support the existence of a close co-localization of VDCC subtypes with the endocytotic proteins clathrin and dynamin in bovine chromaffin cells [81].

The next issue is whether Cav 1.2 or Cav1.3 VDCC has a preferential control on endocytosis. One argument in favor of Cav1.3 is its slower and less complete time-dependent inactivation with respect to CaV 1.2 that would condition the mode of $Ca^{2+}$ entry. The delayed inactivation of Cav 1.3 would favor a slow and prolonged $Ca^{2+}$ entry through the less inactivating L-type channels that could be physiologically relevant for sustaining prolonged $Ca^{2+}$ influxes that support normal endocytosis.

In the study by Rosa et al. (2007), upon the application of a 500-ms DP, the degree of inactivation of each $Ca^{2+}$ channel subtype strongly conditioned the kinetics and the amount of $Ca^{2+}$ entry. Thus, the slow-inactivating L-type channel, which contributes only by about 30% to the initial peak $I_{Ca}$, carried more than half of the total $Ca^{2+}$ entry along the 500-ms depolarizing pulse. Conversely, the fast-inactivating N-type channel that also contributes by about 30% to the initial $I_{Ca}$ peak, only contributed by about 24% to the total $Q_{Ca}$. These data support the idea that a low-rate, non-inactivating $Ca^{2+}$ entry might be more critical to trigger compensatory as well as excess endocytosis [30, 66, 79].

In addition, a pharmacological approach that serves to further slow-down the $Ca^{2+}$ entry through the slow-inactivating L-type calcium channels is based on the use of L-channel activators such as FPL64176 and Bay-K-864. Membrane capacitance recordings and fluorescence imaging with FM-dyes in chromaffin cells have demonstrated that endocytic process is increased in the presence of both agonists without significantly altering exocytosis [14, 81]. The effect of BAY-K-8644 on endocytosis was also studied in the mouse neuromuscular junction, where the vesicle loading with FM2-10 was increased in the presence of the agonist BAY-K-8644 [77]. This finding further supports the hypothesis that L channels are preferentially coupled to the endocytic machinery than the exocytic, and that, not all calcium that enters into the cell through VDCCs have the same function.

## Concluding remarks

By measuring membrane capacitance at the single-cell level, no preferential role of any VDCC subtype in eliciting exocytosis has been found in rat [57] or in bovine CCs [21, 34, 48, 65, 87]. It has been proposed that channel gating and the type of stimuli applied, rather than the possible co-localization of the exocytotic machinery with VDCCs, regulate the exocytosis in chromaffin cells. Thus, a predominant role of P/Q-type channels in regulating the fast release of vesicles when short

stimulation with square depolarizing pulses [8, 9] or trains of action potentials [29] are used, while the slow-inactivating L-type channels would be regulating the sustained or tonic exocytosis when prolonged stimulations are applied [24].

As for exocytosis, it has been proposed that the pattern of stimulation, and therefore, the characteristics of the $Ca^{2+}$ signal generated by the stimulus also influence endocytosis [24]. A predominant role of L-type channels on the regulation of the endocytotic process has been described, but this functional coupling between L channels and endocytosis is related neither to the co-localization of VDCCs and endocytosis proteins nor to the total amount of $Ca^{2+}$ entering the cell through a given subtype of VDCC, suggesting that a low-rate, non-inactivating $Ca^{2+}$ entry through L channels (Cav 1.3) might be more critical to trigger compensatory as well as excess endocytosis [30, 66, 79, 81].

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