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**REVIEW**

# Calcium channels in chromaffin cells: focus on L and T types

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**Abstract**

Voltage-gated $Ca^{2+}$ channels (Cav) are highly expressed in the adrenal chromaffin cells of mammalian species. Besides shaping action potential waveforms, they are directly involved in the excitation–secretion coupling underlying catecholamine release and, possibly, control other $Ca^{2+}$-dependent events that originate near the membrane. These functions are shared by a number of Cav channel types (L, N, P/Q, R and T) which have different structure–function characteristics and whose degree of expression changes remarkably among mammalian species. Understanding precisely the functioning of each voltage-gated $Ca^{2+}$ channels is a crucial task that helps clarifying the $Ca^{2+}$-dependent mechanisms controlling exocytosis during physiological and pathological conditions.In this paper, we focus on classical and new roles that L- and T-type channels play in the control of chromaffin cell excitability and neurotransmitter release. Interestingly, L-type channels are shown to be implicated in the spontaneous autorhythmicity of chromaffin cells, while T-type channels, which are absent in adult chromaffin cells, are coupled with secretion and can be recruited following long-term $\beta$-adrenergic stimulation or chronic hypoxia. This suggests that like other cells, adrenal chromaffin cells undergo effective remodelling of membrane ion channels and cell functioning during prolonged stress conditions.

*Keywords* cAMP/PKA, Cav1 and Cav3 channels, exocytosis, hypoxia, pace-maker current, vesicles.

Voltage-gated $Ca^{2+}$ channels (Cav) play an unquestionable key role in mediating many vital functions. Their importance derives from the fact that they do not only contribute to shaping the action potential and membrane electrical oscillations as do other ion channels, but they also serve as a gate controller of $Ca^{2+}$, the most ubiquitous second messenger. As such, voltage-gated $Ca^{2+}$ channels are implicated in excitation–secretion coupling, excitation–contraction coupling and excitation–transcription coupling (Dolphin 2006).

Voltage-gated $Ca^{2+}$ channels are hetero-oligomeric protein complexes which comprise a main pore-forming $\alpha$1-subunit of 190–250 kDa in association with auxiliary $\beta$-, $\alpha 2\delta$- and $\gamma$-subunits. The $\alpha$1-subunit is composed of four membrane-spanning domains (I–IV) linked together in a single polypeptide chain. Each domain contains six putative transmembrane segments (S1–S6) plus a loop (P) that dips partially into the pore to form the pore lining (Hofmann *et al.* 1999, Catterall 2000). The cytoplasmic loops linking the four domains are structurally important for their interactions with $\beta$-subunits, second messengers, membrane-binding proteins and channel gating. Presently, 10 $\alpha_1$-subunits have been cloned and all of them have specialized functions and distributions. Four members belong to the Cav1 group (L types), three to the Cav2 group (N, P/Q and R types) and three to the Cav3 group (T types). Because of their high threshold of activation, the Cav1 and Cav2 channels are indicated as high-voltage-activated (HVA) channels, while the Cav3 are low-voltage-activated (LVA) channels (Catterall *et al.* 2005).

Structure–function studies have shown that the amino acid sequence of $Ca^{2+}$ channels has positively charged lysine and arginine residues distributed in the S4

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transmembrane segment of each domain, which form the voltage sensor. However, the exact location of the activation and inactivation gates that regulate the open and inactivated states of the pore, are still unknown (Jones 2003).

The adrenal chromaffin cells of many animal species possess, or are capable of expressing, all types of voltage-gated $Ca^{2+}$ channels described above (Garcia *et al.* 2006). In this review, we present and discuss an update of the main properties of L- and T-type channels expressed in chromaffin cells of mammalian species with the purpose of underlying their key role in controlling chromaffin cell excitability and catecholamine release. In the next review, Aaron Fox and collaborators will complement this description by focusing on the properties of Cav2 channel types.

## The L-type channels

L-type $Ca^{2+}$ channels (Cav1) are widely expressed in many tissues and control a number of $Ca^{2+}$-dependent responses in electrically excitable cells. They include four subtypes containing the pore-forming $Ca_v1.1$, Cav1.2, Cav1.3 and Cav1.4-subunits ($\alpha_{1S}$, $\alpha_{1C}$, $\alpha_{1D}$ and $\alpha_{1F}$) with different structure–function characteristics but common blockers: 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines (Catterall *et al.* 2005). Elevation of intracellular $Ca^{2+}$ through L-type channels triggers hormone secretion, cell development, differentiation and apoptosis, and thus the correct characterization of the functioning and modulation of these channel types help understanding key issues of neuroendocrine cell activity and neuronal functioning (Marcantoni *et al.* 2007).

L-type channels belong to the family of HVA $Ca^{2+}$ channels. With the exception of Cav1.3 and Cav1.4, which activate at relatively low voltages (see below); the other Cav1 channels activate at voltages much more positive than resting potential (−30 mV in 5 mM $Ca^{2+}$). Activation is fast and sharply voltage dependent, while inactivation is relatively slow in the presence of $Ba^{2+}$, but speeds up in the presence of $Ca^{2+}$. Deactivation is also fast, ensuring rapid closing of the channels on membrane repolarization to resting levels.

L-type channels are distinguished from the other Cav channels by their high sensitivity to DHPs. DHP antagonists (nifedipine and nitrendipine) reversibly block the channels and help quantifying the amount of L-type channels expressed in a cell, while DHP agonists (Bay K 8644) prolong the open state of the channel, producing slow tail currents near resting potential. As such, DHP agonists allow measuring the activity of single L-type channels in membrane patches, otherwise hardly detectable (Hess *et al.* 1984). L-type channels can often be distinguished from the other $Ca^{2+}$ channels for their cAMP/PKA-mediated upregulation which causes increased mean open times and probability of openings at the single-channel level and increased $Ca^{2+}$ current amplitude in whole-cell recordings (Bean *et al.* 1984).

### *The L-type channels of chromaffin cells*

L-type channels possess several properties that are crucial for the control of neuroendocrine cell activity. First, they are highly expressed and have a primary role in the control of hormone release in pancreatic $\beta$-cells (Yang & Berggren 2006), pituitary glands (Sedej *et al.* 2004) and chromaffin cells (Bossu *et al.* 1991, see Garcia *et al.* 2006 for a review). In addition, their density can be either up- or downregulated by various stimuli, including chronic hypoxia, growth factors, hormones and neurotransmitters (see Marcantoni *et al.* 2007 for a review). Second, L-type channel gating can be effectively inhibited or potentiated by G-proteins coupled receptors (GCPR) (Dolphin 2003). Two mechanisms have been shown to co-exist in chromaffin cells: a membrane-delimited G-protein-dependent inhibition and a remote cAMP/PKA-mediated potentiation (Cesetti *et al.* 2003). Both pathways are activated by autoreleased neurotransmitter molecules and produce opposing effects of comparable entity. Third, L-type channels have a relatively low threshold of activation with respect to the other HVA channels (N, P/Q and R). The threshold of activation is remarkably low for the Cav1.3 isoform (Lipscombe *et al.* 2004), conferring to this channel the ability of pace-making cardiac myocytes (Mangoni *et al.* 2003). This is true also in chromaffin cells that express both Cav1.2 and Cav1.3 (Baldelli *et al.* 2004). Therefore, an open question is how much the two channels contribute to the genesis of action potential firings and how much the G-protein-mediated inhibition and the cAMP/PKA-dependent potentiation may affect the gating of the two channel types. Fourth, L-type channels are tightly coupled with $Ca^{2+}$-activated $K^+$ channels (Prakriya & Lingle 1999). This will condition the shaping of action potential and the frequency of cell firings. Thus, the strict co-localization of bigK (BK) and L-type channels as postulated for RCCs implies that the functioning of other voltage-gated $Ca^{2+}$ channels will be controlled by the activity of L-type channels.

### *Direct and remote L-type channel modulation in RCCs*

L-type channel modulation is heterogeneous and covers a broad spectrum of molecular mechanisms (Marcantoni *et al.* 2007). A major subdivision should include the signalling pathways that are either voltage depen-



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dent or voltage independent. Among the first class should be mentioned: (1) the voltage-dependent facilitation producing L-type current increases following strong and long-lasting pre-pulses described in cardiac (Pietrobon & Hess 1990), neuronal (Kavalali & Plummer 1996) and bovine chromaffin cells (Hoshi *et al.* 1984); and (2) the voltage-dependent and cAMP-mediated phosphorylation, capable of facilitating the L-type channel expressed by a fast phosphorylation reaction favoured by strong depolarizations in chromaffin cells of young cow (Artalejo *et al.* 1992) and skeletal muscle (Sculptoreanu *et al.* 1993) and by the close proximity of PKA to the channel (Gray *et al.* 1997). Both modulatory pathways have been described in past and recent review articles (Dolphin 1999, Carbone *et al.* 2001, Garcia *et al.* 2006, Marcantoni *et al.* 2007).

Here, we will focus on the voltage-independent forms of neuroendocrine L-type channel modulation that include the direct inhibition of L-type channels by G protein-coupled receptors (GPCRs) and the cAMP-mediated potentiation that have autocrine origins and can be back regulated by the material released during secretion.

*Direct inhibition of L-type channels by G proteins.* Neuronal and neuroendocrine L-type channels are effectively inhibited by neurotransmitters through GPCR-mediated pathways. In most cases, the inhibition is voltage independent, but there are examples in which neurotransmitters have no action or the inhibition is even voltage dependent, resembling that of N- and P/Q-type channels (Garcia *et al.* 2006, Fox *et al.*, 2008). The depression causes 20–60% inhibition of the current and there is no delay of L-type channel activation (Baldelli *et al.* 2004).

In the chromaffin cells of adrenal medulla, GPCR-mediated inhibition of L-type channels is fast and voltage-independent. The process is triggered by the same molecules released by the chromaffin granules (ATP, opioids and catecholamines) and produces a scaling down of the current amplitude (Hernández-Guijo *et al.* 1999). The action has been resolved also at the single channel level (Carabelli *et al.* 2001) and is mimicked by ATP, $\mu/\delta$-opioid agonists, adrenaline (A) and noradrenaline (NA) when directly applied on RCCs. It is worth noticing that in BCCs and RCCs (Albillos *et al.* 1996, Hernández-Guijo *et al.* 1999) the L-type current changes its amplitude depending on the flow conditions of cell perfusion. In 'stop–flow' conditions, the current is about half the size recorded during rapid flow in which the secreted material is cleared off the cell, indicating that L-type channel inhibition by neurotransmitters is *autocrine* and acts as negative feedback to control $Ca^{2+}$ fluxes and neurotransmitter release in secretory cells.

An important issue concerning the voltage-independent autocrine inhibition of L-type channels is that facilitatory pre-pulses to +100 mV are ineffective in recovering the depression and the mechanism is direct on the target channel. The action is *membrane delimited* and does not require diffusible messengers. This is supported by the fast onset ($\tau_{on}$ 0.75 s) and offset ($\tau_{off}$ 3 s) of the inhibition during the on and off applications of neurotransmitters (Hernández-Guijo *et al.* 1999) and by the fact that the inhibition is fully defined in cell-attached patches (Carabelli *et al.* 1998, 2001).

*Remote potentiation of L-type channels by the cAMP/PKA pathway.* Cav1.2 channel activity can be effectively potentiated by $\beta$-adrenergic stimulation, direct adenylate cyclase activation or application of membrane diffusible forms of cAMP, as firstly described in cardiac tissues (Bean *et al.* 1984). In BCCs, application of cAMP causes a markedly increased probability of opening which results in an increased L-type channel activity mainly because of a decrease in channel closed times and number of null sweeps rather than an increase in mean open times (Carabelli *et al.* 2001). The cAMP-mediated potentiation is prevented by the PKA inhibitor H89 and proceeds regardless of the presence of the GPCR-mediated inhibition. In RCCs, the cAMP/PKA-induced potentiation of L-type channels is mediated by $\beta_1$-adrenergic receptors ($\beta_1$-ARs), which increase the $Ca^{2+}$ current amplitudes and catecholamine release in autocrinally. $\beta_1$-ARs stimulation in RCCs (Cesetti *et al.* 2003) possesses all the features of the remote action induced by the cAMP/PKA signalling pathway of cardiac cells: (1) is mediated by isoprenaline and blocked by propranolol; (2) is prevented by PKA inhibitors; (3) requires several minutes to reach maximal effects; and (4) is voltage independent and can be induced in cell-attached patch recordings by applying isoprenaline outside the patch-pipette (Carbone *et al.* 2001). The existence of a cAMP/PKA-mediated pathway modulating the L-type channels of chromaffin cells represents a nice example of positive feedback signalling involved in the autocontrol of neurotransmitter release.

Of relevance is the coexistence of two distinct $\beta_1$- and $\beta_2$-AR-activated signaling pathways in RCCs (Cesetti *et al.* 2003). The $\beta_1$-AR cascade acts by selectively upregulating the L-type channel through a PKA-mediated pathway and develops slowly because of its diffusive characteristics, while the $\beta_2$-AR signaling is fast and primarily coupled with pertussis toxin (PTX)-sensitive G-proteins. Figure 1a shows how the two modulatory mechanisms could act either in isolation (left and centre panel) or in parallel (right panel) in which the fast inhibition is followed by the slow potentiation.

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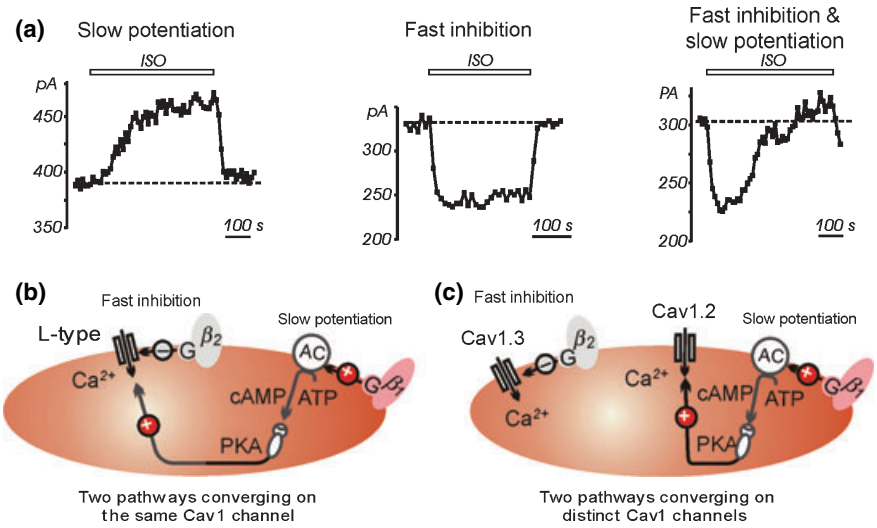


**Figure 1** Inhibition and potentiation of L-type $Ca^{2+}$ currents during isoprenaline (ISO) stimulation in RCCs. (a) Addition of 1 $\mu$M isoprenaline can cause slow potentiation of L-type current amplitudes (*left panel*), fast inhibition (centre panel) or fast inhibition followed by slow potentiation (*right panel*). The symbols are peak current amplitudes measured during a 25-ms step depolarization to +10 mV repeated every 10 s ($V_h$ −40 mV). Adapted from Cesetti *et al.* (2003). (b) Schematic drawing of the signalling pathways in RCCs associated with $\beta_1$- and $\beta_2$-ARs stimulation converging on the same channel type. (d) Same as in (c) but the two pathways are assumed to target Cav1.2 and Cav1.3 separately. Adapted from Marcantoni *et al.* (2007).

### *Two functionally active L-type channels in chromaffin cells?*

The schematic model of Figure 1b assumes arbitrarily that the two opposing mechanisms mediated by $\beta_1$- and $\beta_2$-ARs converge on the same L-type channel but the alternative possibility that the two pathways target two distinct L-type channel isoforms cannot be excluded (Fig. 1c). RCCs and BCCs express Cav1.2 and Cav1.3 channels (García-Palomero *et al.* 2000, Baldelli *et al.* 2004, Benavides *et al.* 2004) and thus the possibility that the direct inhibition by $\beta_2$-AR acts on Cav1.3 and the remote $\beta_1$-AR-mediated potentiation targets Cav1.2 is an interesting possibility that needs to be tested.

The co-existence of two L-type channel isoforms in chromaffin cells, raises important questions on the role that they play in the control of $Ca^{2+}$-dependent exocytosis in neuroendocrine cells which cannot be easily answered because of the lack of selective antagonists capable of distinguishing the two isoforms. A possible approach is that of using knockout mice for Cav1.2 and Cav1.3 channels (Platzer *et al.* 2000) or mutated mice with inborn insensitivity to DHPs ($Cav1.2DHP^{-/-}$) (Sinnegger-Brauns *et al.* 2004), which could furnish indirect information about the properties of the two channels. There are, however, interesting data about the two channels coming from *in vitro* reconstituted cell functions using c-DNA recombinant co-expressed with $\beta$- and $\alpha_2\delta_1$-subunits (Xu & Lipscombe 2001). These studies have highlighted the sharply different voltage-range of activation of the two channel isoforms and other major gating differences. Briefly, the Cav1.3 isoform: (1) activates at −20 mV more negative potentials than the Cav1.2 type; (2) is characterized by fast activation kinetics; and (3) is less sensitive to DHPs. All this indicates that the Cav1.3 is a channel suitable for lowering the threshold of action potential firing and thus furnishing sufficient inward current for pace-making chromaffin cells. On the contrary, the Cav1.2 isoform, which activates at more positive potentials, may be more appropriate for controlling $Ca^{2+}$ entry during the early phase of action potential depolarization or during the repolarization phase.

### *Evidence for a 'low-threshold' L-type channel controlling RCC excitability*

Cultured RCCs express both Cav1.2 and Cav1.3 isoforms, but a selective separation of their biophysical properties has not yet been possible. There are, however, indications that both channels play a critical role in cell excitability and action potential firing. The first evidence is related to the low threshold of L-type current activation (−60 to −40 mV) with an amplitude that is 10% of the total $Ca^{2+}$ current (see Marcantoni *et al.* 2007). For cells with $Ca^{2+}$ currents of 150 pA, this implies that near resting potential (−54 mV) the L-type channels carry about 15 pA and thus is able to produce 15–30 mV depolarization when multiplied by the high input resistance of RCCs (1–2 GΩ). Considering that L-type channels are slowly inactivating in low [$Ca^{2+}$], the sustained L-type current can charge the membrane



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capacitance and initiate spontaneous $Na^+/Ca^{2+}$ spikes with activation threshold at −35 mV. All this agrees with the idea that wild-type Cav1.3 channels display activity that starts from very negative voltages and control the pace making of seno-atrial node cells (Mangoni *et al.* 2003) and neurotransmitter release in cochlear inner and outer hair cells (Michna *et al.* 2003).

The second interesting evidence related to L-type channels in RCCs is their tight coupling with $Ca^{2+}$-dependent BK channels that are highly expressed in BCCs and RCCs (Prakriya & Lingle 1999). BK channels are responsible for the hyperpolarization phase of action potential in these cells and force the action potential to quickly repolarize to about −70 mV. The BK channels of chromaffin cells have, however, a second interesting property. They are fast inactivating, which distinguishes them from the slow inactivating BK channels expressed in most other cells (Orio *et al.* 2002). Fast inactivating BK currents play a crucial role in shaping the action potential repolarization phase and tuning the recruitment of $Na^+$ and $Ca^{2+}$ channels that are responsible for the subsequent slow depolarization phase of spontaneously firing. It is thus reasonable to assume that coupling of L-type with BK channels controls the pace-maker activity in RCCs. As RCCs express L-type channels that are already open at resting potential, it is evident that L-type channels can play the dual role of controlling the action potential shape by activating BK channels and contributing to the pace-maker current that set the frequency of spontaneous firings.

Figure 2a shows an example of spontaneous activity recorded from an RCC in which inhibition of L-type channels exerts a marked effect on cell firing. As shown, the cell is spontaneously active around a mean resting potential of −50 mV and fires regularly at a frequency of 1.3 Hz. Single action potentials are characterized by a slow depolarizing phase that precedes fast depolarization with an overshoot of about +40 mV and then a slower repolarizing phase to −64 mV (Fig. 2b). Addition of 1 $\mu$M nifedipine causes a net prolongation of action potential duration and the disappearance of the undershoot (red trace in Fig. 2b). This is equivalent to blocking the BK channels with the selective blocker paxilline (Gribkoff *et al.* 1996) as shown in Figure 2c, proving unequivocally the tight coupling between BK and L-type channels. Besides affecting the shape of the action potential, nifedipine slows down also the firing frequency (from 1.3 to 0.5 Hz), with further decrement at higher concentrations. The results of Figure 2 reinforce the idea that L-type channels are potentially

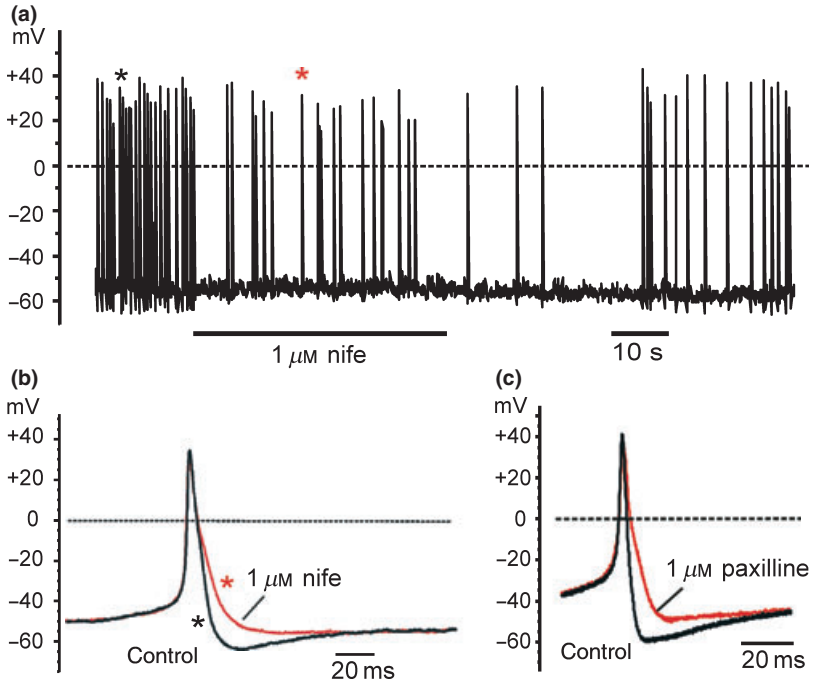


**Figure 2** Spontaneous action potential activity recorded from an RCC in the perforated-patch configuration. The current-clamp recording was achieved by holding the cell at rest without passing current. Data were acquired at 10 kHz and filtered at 1 kHz. The horizontal bar indicates the period of nifedipine application. (a,b) Current-clamp recordings of spontaneous action potentials. In (b) are shown two overlapped action potentials recorded at the time indicated by the asterisks on an expanded timescale; modified from Marcantoni *et al.* (2007). (c) Effects of 1 $\mu$M paxilline on action potentials recorded under the same conditions of panel (a) and (b).

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capable of controlling the firing activity of chromaffin cells because of their low threshold of activation.

### *L-type channels and fast exocytosis in chromaffin cells*

As discussed in recent reviews (Garcia *et al.* 2006, Marcantoni *et al.* 2007), chromaffin cells express different densities of HVA $Ca^{2+}$ channels. Their coexistence at the plasma membrane raises the question of whether all channel types participate in exocytosis and how their expression density and gating properties affect their contribution. In addition, the proportion of $Ca^{2+}$ channels varies widely between animal species and, thus, catecholamine secretion is controlled differently, depending on the more expressed channel types. Concerning the role that L-type channels play in the control of exocytosis, there is unanimous consensus to the idea that they are critically linked to secretion in all mammalian species, even in BCCs in which L-type channels are minimally expressed (García *et al.* 1984).

From all the data reported on $Ca^{2+}$ channels–secretion coupling appears evident that whenever 'strong stimuli' are used (prolonged depolarization with high KCl, sustained applications of ACh or repeated strong depolarizations), the contribution of L-type channels to secretion overwhelms the proportion of $Ca^{2+}$ currents that these channels can carry. L-type channels predominate the secretion despite their contribution to the total current in single-cell experiments is partial or marginal, as in the case of BCCs. A dominance of L-type channel-mediated secretion is thus reported in mammalian chromaffin cells more or less independently of whether intact adrenal glands or cultured chromaffin cells are used (Garcia *et al.* 2006). The reason for this general finding is that during prolonged depolarization (far from physiological conditions) L-type channels are probably more favoured because of their slower time-dependent inactivation and lower steady-state inactivation with respect to the other HVA channels (P/Q, N and R), which inactivate more rapidly and completely during prolonged stimuli. Indeed, when evaluated under voltage-clamp conditions using capacitance changes on single cells, the contribution of L-type channels appear reduced and proportional to the quantity of $Ca^{2+}$ charges carried. As shown in Figure 3, L-type channels contribute to the total secretory response ($\Delta C$) proportionally to the quantity of $Ca^{2+}$ charges ($Q$). There is in fact strict proportionality between $\Delta C$ and $Q$ for L- and non-L-type channels, suggesting similar $Ca^{2+}$ efficiency among HVA channels. These effects are observed in bovine (Engisch & Nowycky 1996, Thiagarajan *et al.* 2004), rat (Kim *et al.* 1995, Carabelli *et al.* 2003) and mouse (Chan *et al.* 2005) chromaffin cells with the exception of adrenal mouse slices in which secretion appears dominated by R-type channels (Albillos *et al.* 2000). Thus, most of the available data favour the idea that, unlike presynaptic terminals in which N- and P/Q-type channels are highly co-localized to the active zone of neurotransmitter release, in chromaffin cells there is no preferential co-localization of any particular $Ca^{2+}$ channel type.

## The T-type channels

T-type $Ca^{2+}$ channels (Cav3) are transient, LVA $Ca^{2+}$ channels that control $Ca^{2+}$ entry during small depolarizations near resting potential. Studies in the past 20 years have lead to a well-defined picture of their functional role in controlling: low-threshold spikes,

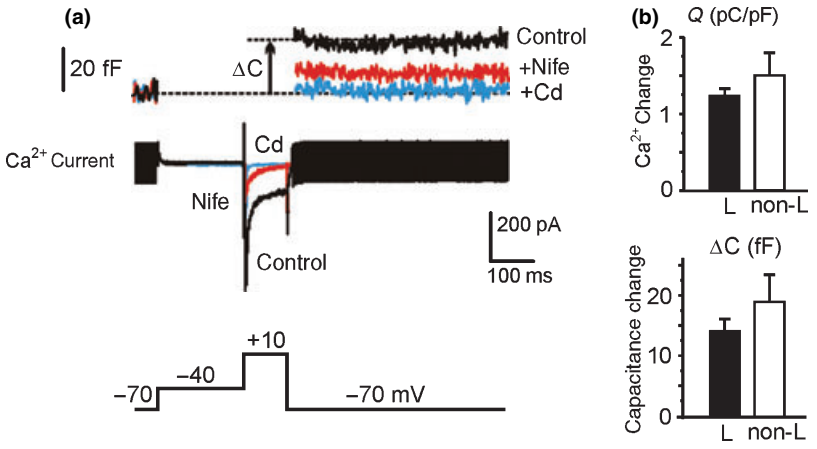


**Figure 3** L-type channel contribution to exocytosis in RCCs. (a) $Ca^{2+}$ currents recorded at +10 mV after incubation with $\omega$-toxins (control) and subsequent addition of 5 $\mu$M nifedipine. To the top are shown the corresponding $\Delta C$ variations. $Ca^{2+}$ currents and exocytosis are fully suppressed by 200 $\mu$M $Cd^{2+}$. (b) Contribution of L- and non-L-type channels to $Ca^{2+}$ charges (top) and exocytosis (bottom) evaluated using the same protocol of panel (a). Adapted from Carabelli *et al.* (2003).



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oscillatory cell activity, muscle contraction, hormone release, cell growth, differentiation and proliferation (Huguenard 1996, Perez-Reyes 2003, Carbone *et al.* 2006, Lory *et al.* 2006). T-type channels are now recognized to be involved in cardiac pace making, slow-wave sleep generation, epilepsy, nociception, vascular tone control, cardiac hypertrophy and fertilization.

T-type channels possess unique biophysical properties (Carbone & Lux 1984, Bossu *et al.* 1985, Fedulova *et al.* 1985, Nowycky *et al.* 1985): (1) channel activation is sharply voltage dependent and starts from low voltages (−50 mV in 5 mM $Ca^{2+}$); (2) inactivation is fast, voltage dependent and insensitive to $Ca^{2+}$; (3) deactivation is particularly slow at potentials near rest; (4) $Ca^{2+}$ and $Ba^{2+}$ are carried with equal efficacy and the permeability of single channel is rather low (5–8 pS at saturating [$Ca^{2+}$]; (5) activation and steady-state inactivation (channel availability) overlap at potentials near rest, uncovering a small fraction of channels that stay open and give rise to an inward 'window current' of 3–20 pA at these potentials; and (6) $Ni^{2+}$ is a good blocker. Taken together, these properties allow the T-type channels to accomplish a number of fundamental physiological functions, such as: (1) lowering the threshold of cell excitability; (2) contributing to the interpulse depolarizing current of pace-making cells; and (3) carrying sufficient steady inward current at rest (window current) to drive $Ca^{2+}$-dependent mechanisms implicated in cell growth and differentiation.

Molecular cloning of T-type channels has provided evidence for the existence of three different pore-forming α1-subunits (α1G, α1H, α1I; Cav3.1, Cav3.2 and Cav3.3) with biophysical profiles similar to the endogenous T-type channels expressed in most tissues (Perez-Reyes 2003). In addition, it furnished the first unequivocal proof that T-type channel α1-subunits have pore structures similar to those of other voltage-gated ion channels and brought new interesting insights into the mechanisms of ion channel selectivity (Talavera & Nilius 2006), activation–inactivation gating (Frazier *et al.* 2001) and new evidence for channelopathies in which Cav3 channels and their splice variants are implicated (Perez-Reyes 2006).

### *The T-type channel of chromaffin cells*

Low-threshold $Ca^{2+}$ currents can be hardly detected in adult chromaffin cells of mammalian species. This is because T-type channels are either absent or weakly expressed in these cells. In adult BCCs, the mRNA encoding for $\alpha_{1G-H}$ is clearly expressed (García-Palomero *et al.* 2000), but functional T-type channels have never been detected (Ceña *et al.* 1983, Artalejo *et al.* 1991, Albillos *et al.* 1993, Carabelli *et al.* 1998), except in one case (Diverse-PierLuissi *et al.* 1991).

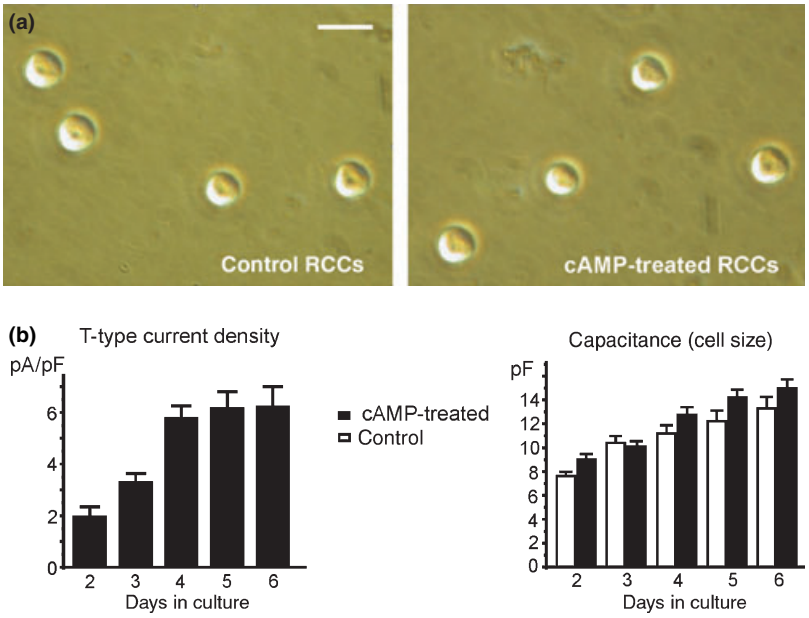


**Figure 4** (a) Photomicrograph of RCCs plated on a plastic dish used for culturing the cells after 4 days in control conditions (*left*) or cAMP-treatment (*right*); scale bar represents 20 μm. (b) Mean T-type current densities (pA $pF^{-1}$) obtained from a large number of RCCs ($39 < n < 56$ and $12 < n < 24$ for cAMP-untreated and cAMP-treated cells). Cells were selected randomly with no specific bias toward their size. To the right is shown the equal membrane capacitance increase with time in culture for cAMP-untreated (open bars) and cAMP-treated RCCs (filled bars). Adapted from Novara *et al.* (2004).

 

T-type channels are nevertheless functionally expressed in embryonic and neonatal RCCs (Bournaud *et al.* 2001, Levitsky & Lopez-Barneo personal communication) and are available in a small percentage of adult RCCs (Hollins & Ikeda 1996, Novara *et al.* 2004, Carabelli *et al.* 2007). Thus, the T-type channel of RCCs behaves like that of neuronal, cardiac and skeletal muscle cells in which they are highly expressed at the embryonic and perinatal period and disappear during the adult stage (Lory *et al.* 2006).

Availability and functionality of T-type channels in chromaffin cells depend critically on cell conditions. In other words, T-type channels can be upregulated during specific stimuli. In RCCs, stress-mimicking agents such as intracellular cAMP, $\beta_1$-AR stimulation and chronic hypoxia induce marked recruitment of T-type channels, requiring either hours or days to reach maximal rates of expressions. Exposures to cAMP produce a marked upregulation of T-type channels after several days (Fig. 4b), while hypoxic conditions (3% $O_2$) requires only 12–18 h. An interesting issue of cAMP and chronic hypoxia treatment is that in both cases the upregulation of $Ca^{2+}$ channels is specific for Cav3.2 ($\alpha_{1H}$) T types, leaving unaltered the density of functioning HVA channels (L, N, P/Q and R). The treatment neither affects the morphology nor the size of the cells (Fig. 4a,c), suggesting that upregulation of T-type channels is not associated with the outgrowth of visible neuritis.

Figure 5 summarizes the main changes that expression of $\alpha_{1H}$ by cAMP elevations or chronic hypoxia produces to $Ca^{2+}$ current recordings. Current traces that usually activate above −20 mV and inactivate weakly in control conditions, are already visible below −40 mV and display the typical transient time course of T-type currents. Activation and inactivation become faster with increasing step depolarizations (Fig. 5, top row). Deactivation, which is fast and changes very little between −50 and −100 mV in control RCCs, becomes 10 times slower at −50 mV and exhibit a slow decaying time course on return to −50 mV when channels are maximally open (bottom row). Finally, the *I–V* curves, which exhibit a single peak at positive voltages associated with predominant HVA channels, display a

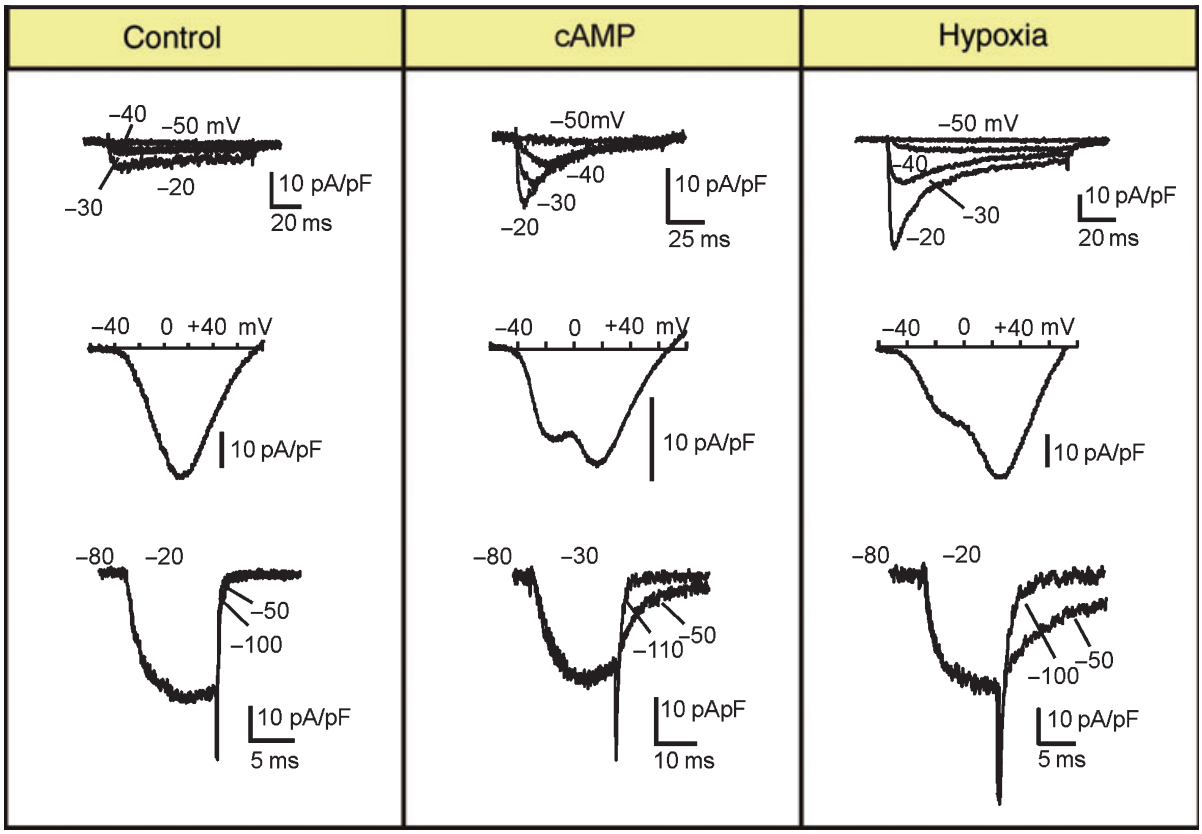


**Figure 5** 'Fingerprints' of T-type currents recorded from cAMP- or hypoxia-treated RCCs. Each box contains recordings of $Ca^{2+}$ currents in control conditions (*left*), after 4 days of cAMP-treatment (*centre*) or overnight incubation in hypoxic conditions (*right*). The recordings in each box are from: (*top*) step depolarizations from −50 to −20 mV, (*middle*) voltage ramp commands and (*bottom*) step depolarizations to −20 or −30 mV followed by a repolarization to −100 or −50 mV. Adapted from Novara *et al.* (2004) and Carabelli *et al.* (2007).



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'low-threshold shoulder' or a clear second peak at negative potentials, depending on the relative contribution of T-type channels to the total current (middle row).

### *Coupling of T-type channels with catecholamine secretion in chromaffin cells*

Expression of $\alpha_{1H}$ channels has two major effects on RCCs: it lowers the threshold of action potential firings (Novara *et al.* 2004) and produces a fast 'low-threshold' secretion that is absent in normal RCCs (Giancippoli *et al.* 2006, Carabelli *et al.* 2007). These two features enhance and broaden the range of RCC functioning. Lowering the threshold of action potential activation may result in an increased frequency of action potentials autorhythmicity, which is expected to produce an increased rate of neurotransmitter release (Zhou & Misler 1995). On the other hand, broadening the range of catecholamine release down to very negative values (−50 and −40 mV) implies that a low rate of secretion could also take place steadily at resting potential. As recently reported (Giancippoli *et al.* 2006, Carabelli *et al.* 2007), the newly expressed $\alpha_{1H}$ channels appear effectively coupled with catecholamine secretion. They produce rapid 'low-threshold' capacitance changes ($\Delta C$) that are associated with the exocytosis of a small number of vesicles ready for release. The amplitude and time course of the capacitance changes associated with T-type channels resemble those induced by HVA channels in normal RCCs (Carabelli *et al.* 2003) and confirm the effective coupling between newly recruited T-type channels and secretory vesicles.

$\alpha_{1H}$ channels have unique properties in controlling secretion that derive from their activation–inactivation gating. Secretion starts at significantly negative membrane potentials (−50 and −40 mV) (Fig. 6) and the voltage dependence of $\Delta C$s and quantity of charge $Q$ do not show 'double peaks' as regards the $I/V$ characteristics. They rather exhibit a net broadening because of the constant contribution of T-type channels to both $\Delta C(V)$ and $Q(V)$ curves over a wide range of voltages (Giancippoli *et al.* 2006). The increased capacitance associated with T-type channels is effectively blocked by low concentrations of $Ni^{2+}$ (50 $\mu$M), which also block $\alpha_{1H}$ T-type currents (Giancippoli *et al.* 2006, Carabelli *et al.* 2007). An interesting issue that remains unsolved is the lack of effective coupling between T-type channels and secretion in embryonic RCCs, even though exocytosis is triggered by HVA channels (Bournaud *et al.* 2001). This suggests that the expression of T-type channels is not *per se* a sufficient condition for coupling with secretory vesicles. The overall mechanism may require the synthesis or the arrangement of molecular components which develop with time in adult tissues.

### *Rate of vesicle release and $Ca^{2+}$ dependence of secretion associated with $\alpha_{1H}$ channels*

Even though $\alpha_{1H}$ channels have unique gating properties, they contribute to secretion with kinetics, $Ca^{2+}$

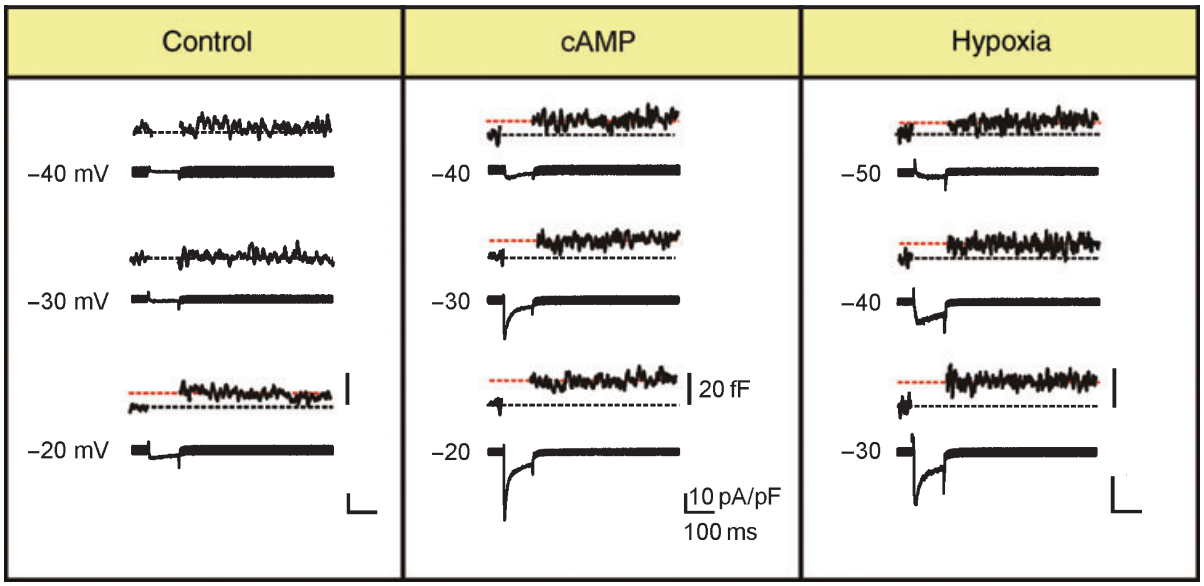


**Figure 6** Depolarization-evoked exocytosis in the presence of cAMP- or hypoxia-recruited T-type channels. Each box shows $Ca^{2+}$ currents and 'low-threshold' capacitance recordings ($\Delta C$) at membrane voltages between −50 and −20 mV recorded in control conditions (*left*), after 4 days of cAMP incubation (*centre*) or 12 h of chronic hypoxia (*right*). Adapted from Giancippoli *et al.* (2006) and Carabelli *et al.* (2007).

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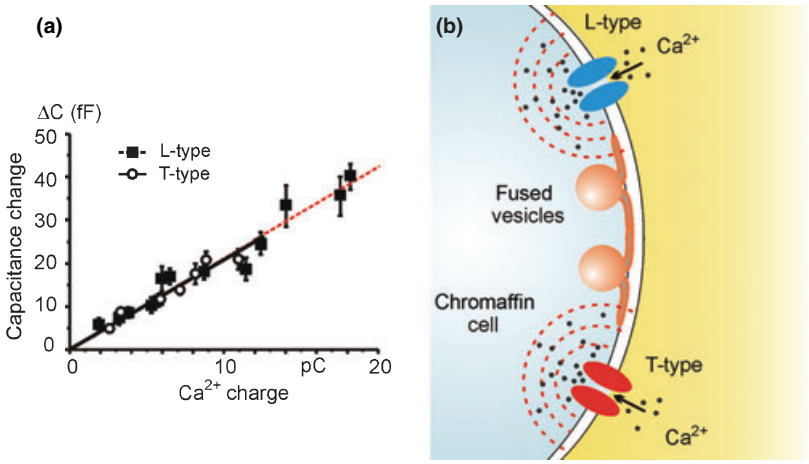


**Figure 7** (a) $Ca^{2+}$ dependence of exocytosis obtained by plotting the mean ΔC increases, measured during capacitative recordings as those illustrated in Figure 6, vs. the corresponding quantity of $Ca^{2+}$ charge. Data are fitted with regression lines of nearly similar slope. The data indicate that T- and L-type channels have nearly identical $Ca^{2+}$ dependence (efficiency). Adapted from Carabelli *et al.* (2003) and Giancippoli *et al.* (2006). (b) Schematic model of $Ca^{2+}$ channel–secretion coupling illustrating similar geometrical distributions of T- and L-type channels near a pool of secretory vesicles.

dependence and probability of release that are comparable with that of HVA channels. This is suggested by several observations. First, the time course of depolarization-evoked exocytosis induced by pulses of increasing durations is exponential, with initial maximal rate of release of 464 fF $s^{-1}$ (equivalent to ~464 vesicles $s^{-1}$) that decreases drastically for very long pulses. ΔC saturates with prolonged depolarizations because of approaching complete mobilization of the immediately releasable pool (IRP) of vesicles and because of the fast inactivation of T-type currents that limits the quantity of charges for pulse >100 ms. Exponential time courses for the depolarization-evoked exocytosis are reported also for the HVA channels, with maximal rates of release of 580 fF $s^{-1}$ (Carabelli *et al.* 2003) and 680 fF $s^{-1}$ (Horrigan & Bookman 1994) for the same cell preparation. A second important observation is that the size of the IRP and probability of release (*P*) estimated by double-pulse protocols are comparable with those associated with HVA channels (Carabelli *et al.* 2003). In particular, *P* is remarkably high (0.6 vs. 0.68 for the HVA channels) indicating a high degree of coupling between T-type channels and release sites.

Finally, the $Ca^{2+}$ dependence of exocytosis evaluated by plotting ΔCs vs. the corresponding quantity of $Ca^{2+}$ charges (~2 fF $pC^{-1}$) is roughly linear and with identical slope when compared with that estimated for L-type channels (Fig. 7a). This indicates that T- and L-type channels couple with the same $Ca^{2+}$ efficiency to exocytosis. It is interesting to notice that T-type channels are effectively coupled with secretion also when they are overexpressed in pheochromocytoma MPC9/3L cell lines that lacks endogenous $Ca^{2+}$ channels but contains secretory vesicles and protein components necessary for exocytosis (Harkins *et al.* 2003). MPC9/3L cells secrete dopamine and catecholamines when $Ca^{2+}$ enters the cell through open $Ca^{2+}$ channels. The interesting aspect of this secretory cell line is that newly expressed α1G T-type channels give rise to $Ca^{2+}$ current densities that are smaller than those associated with α1B N-type channels (400 pA $pF^{-1}$) but produce capacitance increases with comparable efficiency (0.5 fF $pC^{-1}$ for N-type channels vs. 0.6 fF $pC^{-1}$ for the T-type channels).

Taken together, these findings indicate that T-type channels can be effectively coupled with exocytosis in RCCs and they are distributed around the vesicles at the same mean distance of HVA channels. Klingauf & Neher (1997) estimated an average distance of 200 nM for the HVA $Ca^{2+}$ channels from the nearest vesicles in BCCs, and it is likely that the same estimate holds through for HVA and LVA channels in RCCs (Giancippoli *et al.* 2006) (Fig. 7b).

### *Amperometric detections of released catecholamines associated with T-type channels*

Further evidence for an effective coupling of T-type channels with the exocytotic machinery comes from recent measurements with carbon fibre microelectrodes in which hypoxia-recruited $\alpha_{1H}$ channels are shown to induce bursts of exocytotic events during low KCl-induced depolarizations (Carabelli *et al.* 2007). KCl concentrations as low as 2 mM, capable of depolarizing the cells to −65 mV in hypoxic cells, induce



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amperometric spikes associated with catecholamine release which are undetected in normoxic RCCs. Hypoxia-induced amperometric spikes occur at low rates in 2 mM KCl (0.12 Hz) and nearly double in 10 mM KCl (0.27 Hz), when membrane potential reaches −40 mV.

Sustained bursts activity (mean spike frequency 0.64 Hz) is also observed at higher KCl concentrations (15 mM), where mixtures of T- and R-type channels can be activated in hypoxic RCCs incubated with nifedipine, ω-CTx GVIA and ω-Aga IVA to limit the contributions of L-, N- and P/Q-type channels (Fig. 8). Under these conditions, 50 μM $Ni^{2+}$ reduces by about 65% the spike frequency without altering the area, size and time course of amperometric events. This suggests that hypoxia-recruited T-type channels control the release of a pool of secreted vesicles which is indistinguishable from that mobilized by R-type channels, in terms of quantity of released molecules and mechanism of release (Carabelli *et al.* 2007).

The results of Figure 8 are the only available data suggesting the involvement of T-type channels in exocytosis using amperometry and need to be confirmed by combining $Ca^{2+}$ current recordings and carbon fibre detection on the same RCCs, to associate transient T-type $Ca^{2+}$ current recordings with exocytotic events under controlled voltage-clamp conditions.

## Conclusions

Evidence is accumulating on a key role of L- and T-type channels in controlling chromaffin cell excitability and exocytosis. T-type channels are effectively coupled with secretion (Carbone *et al.* 2006) and are recruited following prolonged stress-mimicking conditions (elevated cAMP levels, β-AR stimulation and chronic hypoxia), suggesting that an important membrane ion channel remodelling occurs during pathological stimuli that change cell functioning. Much remains to be done about the role that L-type channels expressed in chromaffin cells (Cav1.2 and Cav1.3) play in the control of their electrical activity and how different modulatory pathways affect the functioning of the two channels. Future studies using KO or mutated mice and modern biophysical techniques will help solving these issues.

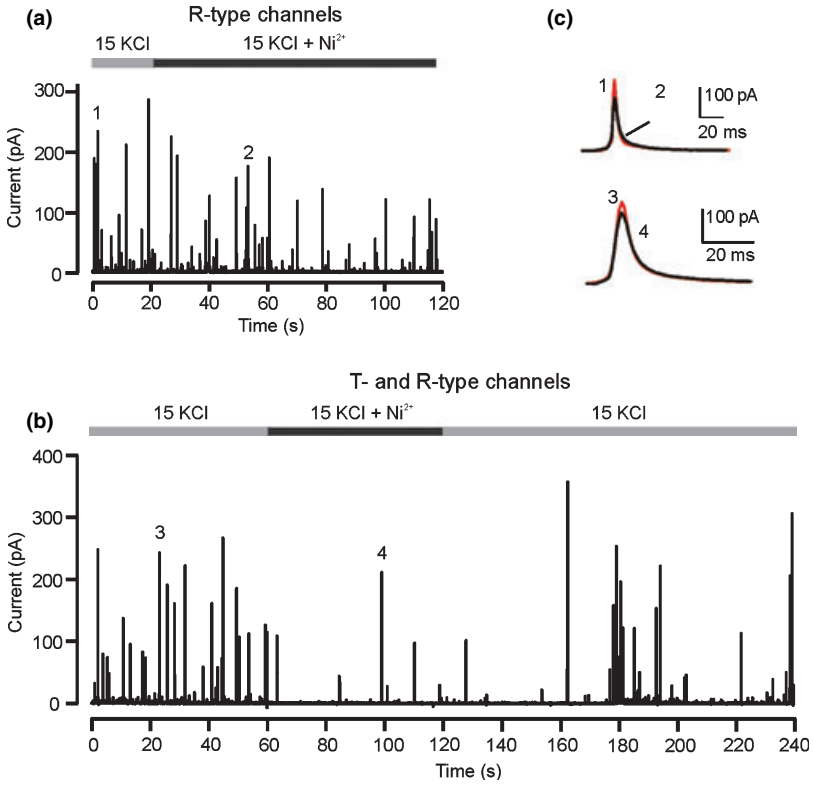


**Figure 8** Amperometric spikes evoked by 15 mM KCl + 1 μM nifedipine, before (grey bar) and during 50 μM $Ni^{2+}$ addition (black bar) in normoxic (a) and hypoxic (b) RCCs. All experiments were performed after ω-toxin incubation and thus in the absence of N-, P/Q- and L-type channels. In (c) is shown the time course of the indicated amperometric events at a more expanded timescale. Notice the reversible action of $Ni^{2+}$ on hypoxic cell. Adapted from Carabelli *et al.* (2007).

 

## Conflict of interest

No conflict of interest.

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