ION CHANNELS

T-type channels-secretion coupling: evidence for a fast low-threshold exocytosis

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Abstract T-type channels are transient low-voltage-activated (LVA) Ca^{2+} channels that control Ca^{2+} entry in excitable cells during small depolarizations around resting potential. Studies in the past 20 years focused on the biophysical, physiological, and molecular characterization of T-type channels in most tissues. This led to a well-defined picture of the functional role of LVA channels in controlling low-threshold spikes, oscillatory cell activity, muscle contraction, hormone release, cell growth and differentiation. So far, little attention has been devoted to the role of T-type channels in transmitter release, which mainly involves channel types belonging to the high-voltage-activated (HVA) Ca^{2+} channel family. However, evidence is accumulating in favor of a unique participation of T-type channels in fast transmitter release. Clear data are now reported in reciprocal synapses of the retina and olfactory bulb, synaptic contacts between primary afferent and second order nociceptive neurons, rhythmic inhibitory interneurons of invertebrates and clonal cell lines transfected with recombinant α_1 channel subunits. T-type channels also regulate the large dense-core vesicle release of neuroendocrine cells where Ca^{2+} dependence, rate of vesicle release, and size of readily releasable pool appear comparable to those associated to HVA channels. This suggests that when sufficiently expressed and properly located near the release zones, T-type channels can trigger fast low-threshold secretion. In this study, we will review the main findings that assign a specific task to T-type channels in fast exocytosis, discussing their possible involvement in the control of the Ca^{2+}-dependent processes regulating exocytosis like vesicle depletion and vesicle recycling.

Introduction

The 25th anniversary of the paper by Hamill et al. [28] is an appropriate occasion to remind that among the many important goals achieved by the "patch-clamp technique", there is also the detailed description of the biophysical features of LVA T-type channels. It was indeed few years after the publication of the famous Pfluegers Archiv's article [28] that several groups could identify and describe the unique properties of LVA channels [2, 4, 6, 11, 12, 22, 48, 50].

LVA channels belong to the family of voltage-gated Ca^{2+} channels, which also includes the HVA channel types (L, N, P/Q, and R). These latter activate at relatively higher voltages with respect to T-type channels and possess distinct structural and functional properties (see [14]). T-type channels are ubiquitously expressed and sustain key physiological functions like low-threshold spikes (LTS) generation, neuronal and cardiac pacemaking activities, muscle contraction, hormone release, cell growth and differentiation [33, 66, 72, 73]. Most of these functions derive from the unique properties that LVA channels are shown to possess: (1) they activate and desensitize (inactivate) at unusually negative voltages (>−70 mV), (2) exhibit transient kinetics due to fast and complete inactivation on sustained depolarization and deactivate slowly on repolarization, (3) are equally permeable to Ca^{2+} and Ba^{2+}

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and have small single channel conductance, (4) outlast membrane-patch excision and are preferentially blocked by low doses of Ni^{2+} and mibefradil.

Following this preliminary biophysical and pharmacological characterization, T-type channels have been widely investigated in nearly all tissues to uncover their functional role and molecular identity [52]. The progress of these studies, however, has been often biased by the lack of high-affinity blockers which limited the analyses to narrow ranges of voltages and, in some cases, may have caused serious underestimates of their role in specific physiological functions (see below). A sharp improvement to this approach was provided by the molecular cloning of three different pore-forming α_1 subunits (α_{1G}, α_{1H}, and α_{1I} also named as $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$, respectively) with biophysical properties similar to the endogenous T-type channels [17, 53]. This opened a new era of investigations, particularly for the structure–function studies, which have brought important new insights into the mechanisms of ion selectivity and activation–inactivation coupling [52] (see also [10]).

At present, T-type channels are recognized to play a critical role in many physiological functions in which a low-threshold Ca^{2+} entry is required to trigger or sustain specific cell activities. This is particularly true for the generation of LTS, oscillatory cell activity, and hormone secretion in cells lacking a vesicular apparatus. The critical role of T-type channels on important physiological functions is strengthened by the fact that either recruitment, overexpression, or malfunctioning of these channels is reported in a number of pathologies, including cardiac hypertrophy [65], hypertension [43], heart failure [16], absence epilepsy [68], neurogenic pain [1], and Parkinson's disease [73]. Most recently, T-type channels are shown to control the vesicular release of neurotransmitters in neurons and neuroendocrine cells [42, 51], and very recent data indicate that LVA channels are involved in fast catecholamine release in adrenal chromaffin cells [9, 25].

In this review, we will focus on the role that T-type channels play in the control of fast vesicular exocytosis. This aspect has been largely overlooked in the past and, given its importance, may turn of broad interest for future investigations. As the contribution of T-type channels to exocytosis derives mainly from channels located at distal dendrites and spines, the progress in this field will rely on whether optical techniques for recording Ca^{2+} signaling and electrophysiological detection of vesicle secretion will improve their sensitivity to reveal Ca^{2+} signals and exocytic events in narrow regions of neuronal terminals. T-type channels are known to play also a main role in the control of hormone release from cells lacking secretory vesicles and exocytotic mechanisms. This aspect, however, will not be treated in this study because there are already excellent articles and up-to-date reviews covering this issue [40, 55, 58, 61].

Transmitter release and T-type channels in neuronal terminals

A brief overview

Pharmacological studies and immunocytochemical characterization of Ca^{2+} channel distribution suggest that N- and P/Q-type channels are the predominant species controlling synaptic transmission in central neurons [54]. The two Ca_v2 channel types bind to syntaxin and synaptotagmin through a "synprint" region [45] and are closely associated to the SNARE complex. They mediate vesicle docking, fusion, and neurotransmitter release in highly specialized presynaptic regions (active zones). Colocalization of N- and P/Q-type channels to the active zones ensures high Ca^{2+} concentrations and fast synchronous synaptic responses during action potential-evoked neurotransmitter release. In this way, probability of release (p) associated to N- and P/Q-type channels is relatively large when $[Ca^{2+}]_i$ is elevated above 10 μM, ensuring high rates of vesicle release during rapid responses (~300 vesicles/ms; [57]). There are, however, exceptions to this general rule suggesting that also other voltage-gated Ca^{2+} channels (R-, L-, and T-type) play a role in the control of presynaptic Ca^{2+} levels in a variety of neurons.

In cerebellar parallel fibers [44] and hippocampal CA3-CA1 synapses [70], a conspicuous fraction of $[Ca^{2+}]_i$ persists in the presence of ω-CgTx, ω-Aga-IVA, and nifedipine, suggesting that either R- or T-type channels may contribute as well to Ca^{2+} elevations in these terminals during synaptic transmission. Evidence for a contribution of R-type channels on glutamate release is reported at the calyx of Held [71], and R-type channels are shown to replace P/Q-type channels at the mouse neuromuscular junction in KO mice lacking P/Q-type channels [67]. L-type channels are shown to control glutamate release in rod photoreceptor terminals [69] and to colocalize at the hot spots of bipolar cell terminals [5]. L-type channels are expressed also in AII amacrine cells and localized at the distal dendrites where synaptic transmission takes place [27]. The most noteworthy is that L-type channels regulate transmitter release in single boutons of cultured rat hippocampal neurons [46] and are involved in presynaptic Ca^{2+} accumulation during high-frequency activity in GABAergic hippocampal neurons [36]. This helps maintain high rates of vesicle release during tetanic stimulation and to enhance the probability of transmitter release during posttetanic periods. In cerebral cortex, L-type channels control short-term plasticity of GABA release during

presynaptic firing rates in the gamma frequency range (40 Hz) [37]. The action is likely mediated by second messengers, which may activate various kinases like myosin light-chain kinases, controlling vesicle recycling and mobilization from a vesicle reserve pool.

To date, there is also increasing evidence that T-type channels mediate neurotransmitter release in a number of neurons. They control the response to graded depolarizations in rod bipolar neurons [51, 60], are involved in neurotransmitter release in dendrodendritic reciprocal synapses between granule cells (GCs) and mitral/tuftet cells of olfactory bulbs [20, 21], control spike-mediated and graded synaptic transmission between oscillatory heart interneurons of leech [34], and regulate the frequency of spontaneous excitatory postsynaptic currents (mEPSCs) in nociceptive neurons [3]. Looking closely at these reports, it appears evident that T-type channels play a role in those neurons that either use graded potentials for signal transmission or display Ca^{2+} signals at the synaptic terminals in dendrites and spines that are strongly dependent on the holding membrane potential. In all cases, the indication is unambiguous and brings indisputable evidence in favor of a direct role of T-type channels on neurotransmitter release.

T-type channels control GABA release in invertebrate inhibitory interneurons

In the leech, the neuronal network generating rhythmic heartbeats includes two segmental bilateral pairs of reciprocal inhibitory interneurons. The bilateral neurons are active and alternate bursts, which inhibit one another via graded and spike-mediated transmission. T-type channels mediate the graded response, whereas L-type channels control the spike-mediated transmission [41]. Simultaneous recordings of presynaptic Ca^{2+} currents, presynaptic changes of Ca^{2+} fluorescence, and postsynaptic inhibitory postsynaptic currents (IPSCs) show that transient T-type currents at −35 mV produce sustained $[Ca^{2+}]$ increases at the fine neuritic branches near the region of synaptic contact. The $[Ca^{2+}]$ increases at the neuronal endings are closely associated to transient IPSCs [34]. There is nice correlation between presynaptic Ca^{2+} increases and rise of postsynaptic conductance and an even better correlation between the transient time course of T-type currents and transient IPSCs, proving an unequivocal link between LVA channels activation and graded postsynaptic response in these inhibitory interneurons. T-type channels in leech heart interneurons also control the background $[Ca^{2+}]$ changes that modulate the strength of spike-mediated synaptic transmission [35]. In this view, T-type channels appear critical to the control of neurotransmitter release, which set the oscillatory rhythms of leech heartbeats.

T-type channels regulate GABA release in olfactory bulbs

T-type channels have a unique functional role also at the large spines of GCs that form dendrodendritic reciprocal synapses with mitral and tufted cells of olfactory bulbs. As shown by several groups, the distal spines of GCs possess presynaptic (transmitter-releasing) and postsynaptic (transmitter-receiving) elements. These terminals express sufficient densities of T-type channels to modulate transmitter release [20]. The presence of T-type channels is evident by two-photon Ca^{2+} imaging measurements showing that Ca^{2+} transients at the spines and adjacent dendrites of GCs are strongly dependent on membrane potential: decreasing their size with depolarization and increasing with hyperpolarization. Both the voltage dependence and time course of inactivation of Ca^{2+} transients are consistent with the known properties of T-type channels, and Ca^{2+} signals appear markedly reduced by applications of Ni^{2+} (100 μM) and mibefradil (1–10 μM). Mibefradil reduces also the action potential-evoked synaptic transmission from granule to mitral cells. In this case, action potentials are evoked by extracellular stimulation, while inhibitory postsynaptic potentials (IPSPs) are recorded in whole-cell mode at the soma of mitral cells. Under these conditions, small IPSPs with short latency and slow decay time constant can be recorded in a number of mitral cells. The IPSPs are fully blocked by bicuculline and strongly attenuated by 10 μM mibefradil, suggesting that they are mediated by $GABA_A$ receptors and that mibefradil-sensitive Ca^{2+} channels contribute to at least half of the global inhibition of mitral cells induced by GC stimulation. These findings uncover a dual role for T-type channels on both the control of GABA release from dendritic spines and the graded regulation of GC-mediated lateral inhibition in the olfactory bulb. A graded "low-threshold" control of GABAergic synaptic release mediated by T-type channels may represent a new way to gate the entry of olfactory information to the cortex [21].

T-type channels regulate the frequency of mEPSCs in nociceptive neurons

Increasing evidence indicates that T-type channels play a key role in the control of nociceptive signaling [1]. LVA channels are highly expressed in mammalian sensory neurons [13, 22, 59], and upregulation of their current by reducing agents like DTT or endogenous L-cysteine induces thermal hyperalgesia in animal models of neuropathic pain [64]. T-type channels are also directly involved in the control of synaptic activity between primary afferents and second-order nociceptive neurons of the dorsal horn of the spinal cord by controlling the frequency of spontaneous EPSCs (mEPSCs) mediated by AMPA/kainate receptors

[3]. T-type channel blockers like Ni^{2+} (100 μM), mibefradil (5 μM), and La^{3+} (5 μM) are very effective in drastically reducing the frequency of mEPSCs without significantly affecting the latency and amplitude of action potential-evoked EPSCs. In contrast, HVA channel blockers like Cd^{2+} (50 μM) and ω-CgTx-GVIA (1 μM) can fully block the EPSCs without affecting the frequency of mEPSCs. Worth mentioning is the observation that small depolarizations induced by raising $[K]_o$ to 10 mM produce a marked increase of mEPSCs frequency, fully prevented by 5 μM mibefradil. This indicates that spontaneous glutamate release near resting conditions is effectively controlled by T-type channels in nociceptive neurons. Because mEPSCs occur often enough to influence postsynaptic neuronal excitability, it is evident that the availability of low-threshold T-type channels alters the excitability level of nociceptive neurons. Excitatory transients occurring at a rate of 10–30/s, each lasting 10–30 ms, can easily coincide with the evoked activity of other terminals and summate to threshold levels for a postsynaptic afferent neuron, lowering the threshold of pain sensation.

T-type channels contribute to fast glutamate release in retinal bipolar cells

In mammalian retina, T-type channels are coexpressed with L-type channels in rod bipolar cells [30] and contribute to neurotransmitter release at the reciprocal synaptic contacts that bipolar cells form with A17 and AII amacrine cells [51, 60]. Several lines of evidence support a functional role and a tight coupling of T-type channels to glutamate release in this preparation. A first evidence concerns the high density of T-type channels expressed in these cells compared to L-type channels [51, 60]. The two channel types give rise to Ca^{2+} currents of comparable amplitudes (60 pA at −40 mV for the LVA and 40 pA at −10 mV for the HVA, [51]), which implies rather similar densities if correcting for the different driving force, single channel conductance, and assuming equal open probability for the two channels. A second evidence concerns the strict voltage-dependent correlation existing between LVA currents and membrane capacitance changes in isolated rod bipolar cells [51] (see below). Finally, there is strong correspondence between the time course of presynaptic Ca^{2+} currents of bipolar cells and the fast glutamatergic responses recorded in synaptically interconnected amacrine cells [60].

At the synapses formed by bipolar and AII cells, the T-type channels recruited by strong hyperpolarization (−100 mV) give rise to a transient low-threshold presynaptic Ca^{2+} current that markedly increases the amplitude of fast EPSCs with little changes to its transient time course [60]. Indeed, the EPSCs recorded in AII cells are transient also when L-types are the only Ca^{2+} channels contributing to the presynaptic current, but the contribution of the T-type current almost doubles the amplitude of AMPA-mediated EPSCs. T-type currents are almost four times the L-type currents elicited from −50 mV holding potential and have all the hallmarks of LVA channels: low-threshold of activation, fast inactivation, and slow deactivation.

These findings suggest unequivocally that, when available, T-type channels contribute to neurotransmitter release with the same efficiency of HVA channels. The slight delay in the synaptic response (~5 ms) introduced on step depolarizations from very negative holding potentials (−100 mV) does not seem to be attributable to the contribution of T-type channels. In addition, the recruitment of T-type channels in retinal bipolar cells is already effective at holding potentials around −80 mV [51], and synaptic activity at these potentials, when T-type channels are available and L-type channels are blocked by 10 μM nimodipine, appears extremely fast. This is proved by the short delay (<10 ms) existing between the raising of T-type current at −40 mV and the appearance of reciprocal GABAergic synaptic activity in identified bipolar cells of retinal slices [51]. Notice that the reciprocal inhibitory responses in bipolar cells, which appear as rapid fluctuations on top of the conventional T-type current, are always preceded by an excitatory synaptic response from bipolar to amacrine cells. Under these conditions, the true synaptic delay between T-type channel activation and glutamate release is limited to few milliseconds and is thus comparable to the synaptic delays associated to N- and P/Q-type channels.

T-type channels–secretion coupling viewed through high-resolution capacitance changes

Fast exocytosis and T-type currents in retinal bipolar cells

Strong evidence that T-type channels mediate rapid neurotransmitter release in retinal bipolar cells comes from the work of Pan et al. [51] in which glutamate release is directly measured by membrane capacitance increases (ΔCs) associated to increases of membrane surface area during presynaptic vesicle fusion [47]. In isolated bipolar cell, activation of T-type currents results in a net Ca^{2+} influx in the presynaptic endings and fast exocytosis that is detected as ΔC increases. Capacitance increases induced by Ca^{2+} currents associated to 500-ms depolarizing pulses to −10 mV are in the order of 13 fF (corresponding to the release of 169 synaptic vesicles, if 1 fF is associated to the release of 13 vesicles with 50 nm mean diameter) and are fully abolished by 4 mM Co^{2+} or by replacing intracellular EGTA with BAPTA. Inhibition of exocytosis by BAPTA proves unequivocally that capacitance changes evoked on

step depolarizations are induced by elevations of intracellular Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels (T- and L-types). Consistent with this is also the observation that the ΔCs increase to about 20 fF when lower EGTA concentrations (0.5 mM) are used in place of normal EGTA (5 mM). Capacitance increases occur also at very low membrane voltages (−40 to −30 mV) where LVA channels can be recruited by holding the axon terminals at −85 mV. These "low-threshold" capacitance changes are in the order of 7 fF and are fully blocked by 10 μM mibefradil. Nimodipine (10 μM) has no action on them but drastically reduces the ΔCs at −10 mV, which are mainly associated to the L-type channels expressed in rod bipolar cells. The work of Pan et al. [51] thus furnishes the most direct evidence that T-type channels can indeed support fast exocytosis in synaptic terminals subjected to graded depolarizations.

How fast do T-type channels mediate the exocytotic response? Presently, there are no data available on capacitance measurements at intervals below 500 ms, and thus it is impossible to make precise estimates of the initial rate of vesicle release and Ca^{2+} dependence of exocytosis in retinal bipolar cells. However, Pan et al. showed very clearly that intracellular Ca^{2+} fluxes at the presynaptic terminals of bipolar cells raise with time constants in the order of 100–200 ms, and thus it is possible that capacitance changes will follow this speed. In addition, as pointed out above, LVA currents lasting 50 ms can evoke reciprocal inhibitory currents in bipolar cells of retinal slices. A transient fluctuating current associated to the activity of $GABA_A$ receptors is already evident after 10 ms from the start of the current [51], indicating that LVA channels are capable of stimulating neurotransmitter release within very short times. Thus, it is likely that T-type channels may trigger neurotransmitter release in bipolar cell and contribute to the overall retinal information processing.

Capacitance changes in pheochromocytoma cell lines expressing recombinant α_{1G} Ca^{2+} channels

T-type channels are effectively coupled to secretion also when they are overexpressed in a mouse pheochromocytoma cell line (MPC9/3L) that lacks endogenous Ca^{2+} channels but contains secretory vesicles and the protein components necessary for exocytosis [29]. MPC9/3L cells secrete dopamine and catecholamines when Ca^{2+} enters the cell through exogenously applied Ca^{2+} ionophores or when heterologous Ca^{2+} channels open on step depolarizations. The interesting aspect of this secretory cell line is that newly expressed α_{1G} T-type channels give rise to Ca^{2+} current densities (70 pA/pF) that are significantly smaller than those associated with α_{1B} N-type channels (400 pA/pF) but produce capacitance increases with comparable

efficiency. In fact, dividing the capacitance changes for the quantity of Ca^{2+} charges gives mean efficiencies of 0.5 fF/pC for the N-type channel vs 0.6 fF/pC for the T-type channel and similar values for the α_{1C} L-type channel that is expressed at even lower densities. T-type channels are also capable of inducing cumulative capacitance increases during brief trains of 5–10 depolarizations of 10 Hz, suggesting that they can sustain marked exocytosis also when activated at frequencies comparable to sympathetic stimulation (2–20 Hz).

The rationale for these observations is that in MPC9/3L cells, all Ca^{2+} channels can functionally couple to release sites and control rapid exocytosis. Thus, the presence of protein components such as syntaxin and SNAP-25, which are essential components of the SNARE complex mediating vesicle fusion, and the expression of high densities of N-type channels do not produce preferential couplings between Ca^{2+} entry and vesicle release. Also, the fact that N-type channels possess a "synprint region", which is absent in T- and L-type channels and is necessary for binding of syntaxin, appears not to be a critical element for mediating fast vesicle depletion in MPC9/3L cells.

T-type channels and fast capacitance changes in rat melanotropes

Indications that T-type channels are effectively coupled to large dense-core vesicles (LDCVs) secretion come also from ΔC measurements in rat melanotropes. These cells express all types of voltage-gated Ca^{2+} channels (HVA and LVA), which fully control the release of predocked LDCVs during action potential stimulation [42]. T-type channels in rat melanotropes contribute to a small fraction of the total current and produce a mean ΔC increase of about 7 fF during 40 ms step depolarization that is only about half the ΔC associated to HVA channels (L-, N-, P/Q-, and R-type). Normalizing the capacitance changes for the quantity of Ca^{2+} ions entering the cells through open channels (Ca^{2+} current integral), it can be shown that T-type channels control secretion with the same efficacy of HVA channel types. The immediate significance of this result is that T-type channels are coupled to LDCVs with the same strength of all the HVA channels. It is interesting to notice, however, that in rat melanotropes as in chromaffin cells, voltage-gated Ca^{2+} channels are not colocalized with LDCVs. This implies some delay between Ca^{2+} entry and vesicle secretion, which may result in poor coupling between single action potentials and exocytosis if the action potential lasts a few milliseconds and Ca^{2+} must diffuse considerable distances (200–300 nm) to reach docked vesicles [38]. This however is not the case of rat melanotropes in which action potentials last very long (50–300 ms) and Ca^{2+} entry persists for the entire duration
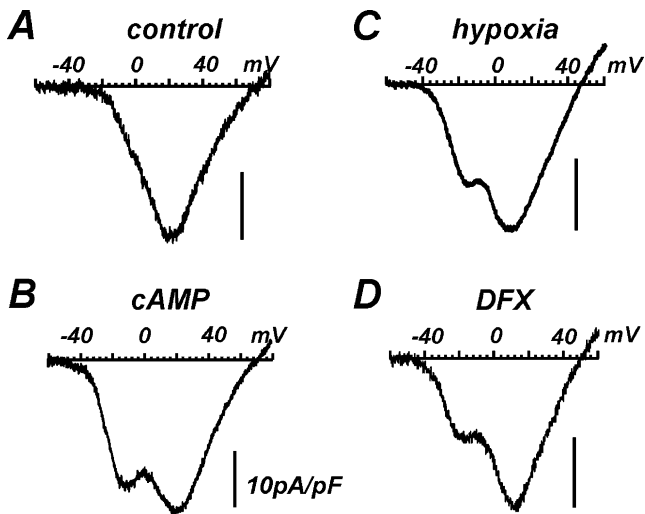
A control
B cAMP
C hypoxia
D DFX

10pA/pF

Fig. 1 Recruitment of α_{1H} T-type currents after exposure to cAMP, chronic hypoxia, and the hypoxia mimicking agent deferoxamine (DFX). **a, b** Whole-cell Ca^{2+} currents recorded in 10 mM Ca^{2+} during a ramp command from −60 to +60 mV from −80 mV holding potential in control RCCs and from RCCs pretreated with 200 μM pCPT-cAMP and incubated for 5 days. Notice the second "low-threshold peak" associated to the α_{1H} T-type currents recruited after cAMP treatment (see [49]). **c, d** Ca^{2+} currents recorded in patch-perforated conditions (5 mM Ca^{2+}) during a ramp command from −60 to +60 mV (−80 mV holding potential) in RCCs exposed overnight to a 5% CO_2 and 3% O_2 atmosphere (hypoxia) or to 300 μM deferoxamine that activates hypoxia-inducible transcription factors. Notice the appearance of a "low-threshold peak" in both cases. Expression of α_{1H} T-type channels during hypoxia has been reported also in PC12 cells [19]

generation. In the case of T-type channels, they activate around −60 mV and contribute to the slow initial phase of depolarization and to the "low-threshold" component of exocytosis with the same efficacy of HVA channels. This is shown by simultaneously recording capacitance changes and Ca^{2+} currents while clamping the cell with truncated action potentials to separate different parts of action potential stimuli. T-type channels produce small ΔCs of 2.2 fF (equivalent to the release of two LDCVs) that normalized for the quantity of Ca^{2+} charges furnishing a Ca^{2+} dependence similar to that of HVA channels. This ensures that exocytosis takes place over the entire duration of the action potential with the same efficacy regardless of the type of open Ca^{2+} channel.

Capacitance changes associated to T-type channels in rat chromaffin cells

As reported by various groups, T-type channels are either absent or weakly expressed in bovine and rat chromaffin cells (RCCs) [15, 23, 24, 31] but can be expressed when RCCs are exposed to cAMP for several days [25, 49], after overnight incubation in hypoxic conditions (3% O_2) or in the presence of the hypoxia mimicking factor deferoxamine (DFX) (Fig. 1; see also [9] and [19]). Under these conditions, newly expressed α_{1H} T-type channels appear to be effectively coupled to catecholamine secretion and produce rapid exocytic events that closely resemble those produced by HVA channels [25]. The functional coupling between T-type channels and the exocytotic apparatus of chromaffin cells allows precise measurements of the elementary parameters controlling secretion, which are
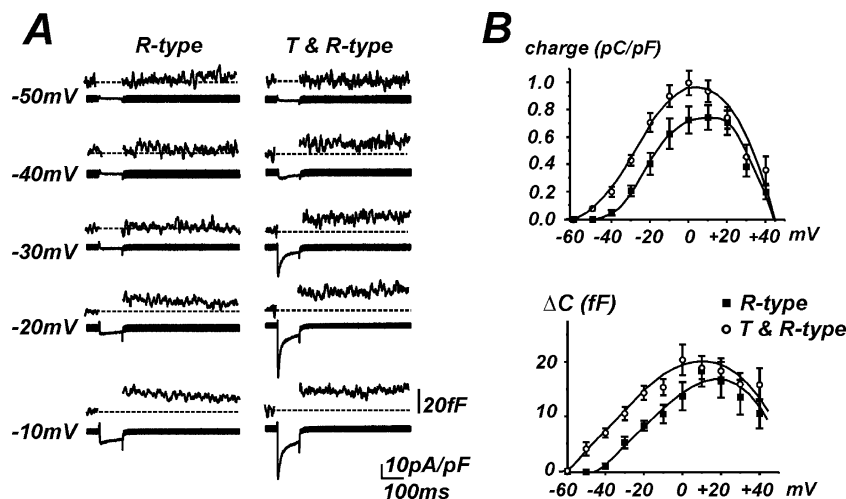
of the depolarization. Under these conditions, all voltage-gated Ca^{2+} channels can contribute to secretion by different degrees during the various phases of action potential

A R-type   T & R-type
-50mV
-40mV
-30mV
-20mV
-10mV

20fF

10pA/pF
100ms

B charge (pC/pF)
1.0
0.8
0.6
0.4
0.2
0.0
-60 -40 -20 0 +20 +40 mV

ΔC (fF)
■ R-type
○ T & R-type
20
10
0
-60 -40 -20 0 +20 +40 mV

Fig. 2 "Low-threshold" capacitance changes associated with T-type channels. **a** Secretory responses evoked by R- and T-type currents from a control RCC and after exposure to pCPT-cAMP recorded in patch-perforated mode. RCCs were incubated with ω-toxins, and nifedipine was in the bath. **b** Quantity of Ca^{2+} charge and

corresponding secretion measured as ΔC increases at the potentials indicated. The quantity of charge was calculated as the time integral of the current entering during the 100 ms depolarization (adapted from [25])
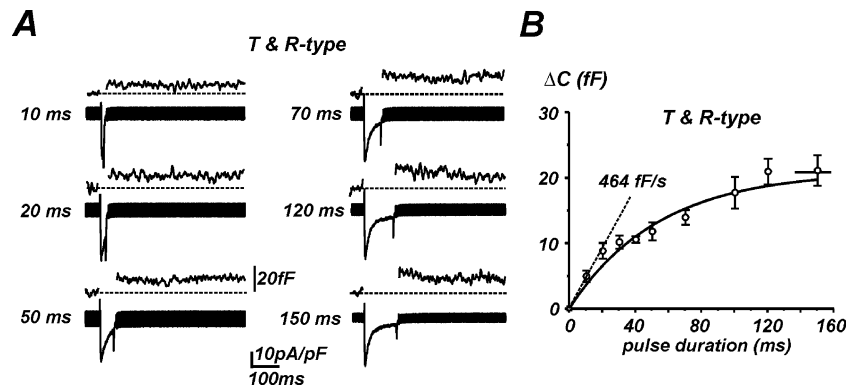
Fig. 3 T-type channels and maximal rate of vesicle depletion in RCCs. **a** RCCs were pretreated with ω-toxins and nifedipine and depolarized with pulses of increasing length (from 10 to 150 ms) at −20 mV to progressively deplete the pool of vesicles ready for release. **b** Plot of ΔC increases vs the corresponding pulse length. Data are averaged from 20 cAMP-treated cells as the one of panel **a**. The *solid curve* is an exponential fit with $\tau = 54$ ms and maximal value indicated by the asymptote (21 fF). The *dashed line* drawn to fit the first two points at 10 and 20 ms has a slope of 464 fF/s and indicates the maximum rate of vesicle depletion (adapted from [25])

worth discussing in this study. In addition, chromaffin cells express all kinds of voltage-gated Ca^{2+} channels (L-, N-, P/Q-, R-, and T-type) and thus represent an ideal system for comparing the role of each channel type to secretion.

α_{1H} T-type channels have unique properties in controlling secretion, which derive from the peculiar characteristics of their activation–inactivation gating. First, secretion starts at significantly negative membrane potentials (−50 and −40 mV), as soon as T-type channels carry sufficient Ca^{2+} ions inside the cell to trigger exocytosis (Fig. 2a). Second, the relationships of capacitance changes and quantity of charge vs voltage [$\Delta C(V)$ and $Q(V)$] do not show "double peaks" as for the I/V characteristics [11–13] but rather a sharp broadening due to the constant contribution of T-type channels to both $\Delta C(V)$ and $Q(V)$ over a wide range of voltages (Fig. 2b). This derives from the weak voltage dependence of the quantity of charges passing through transiently open T-type channels. In fact, the sharp increase of the peak current with increasing voltages is followed by an increased rate of inactivation that

limits the quantity of Ca^{2+} charges during pulses of 100 ms. Third, the increased capacitance associated to T-type channels is effectively blocked by low concentrations of Ni^{2+} (50 μM), which also block α_{1H} T-type currents [39]. Fourth, long-term cAMP treatment, chronic hypoxia, or DFX recruits T-type channels without altering HVA channels' density and their related secretion [25].

Rate of vesicle release, Ca^{2+} dependence, and size of immediately releasable vesicles coupled to α_{1H} T-type channels

Even if α_{1H} channels have unique gating properties, they contribute to secretion with kinetics, Ca^{2+} dependence, and probability of release that are comparable to that of HVA channels normally expressed in RCCs. This is suggested by several observations. First, the time course of depolarization-evoked exocytosis is exponential, with an initial maximal rate of release of 464 fF/s (equivalent to ~464 vesicles/s if assuming 1 fF as the unitary capacitance
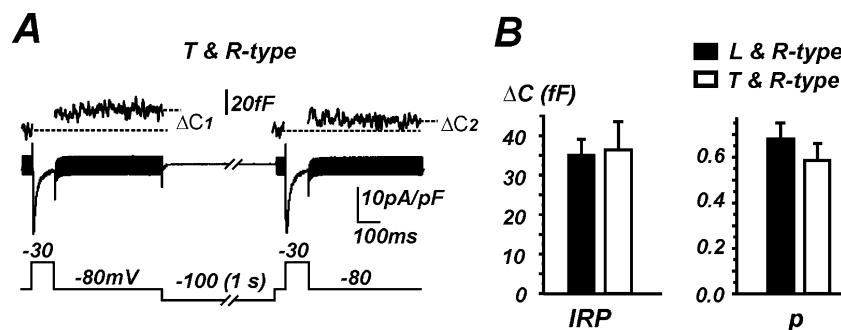

Fig. 4 T- and L-type channels mobilize the same IRP and induce comparable probability of release. **a** Double-pulse protocol for estimating the IRP size and the probability of release (*p*). Two depolarizations of 100 ms at −30 mV were applied consecutively after a 1-s interval to −100 mV as illustrated. Each pair of depolarization was repeated at least three times, and values of ΔC_1 and ΔC_2 were averaged for each cell. **b** Bar histograms indicate the calculated values of $IRP = (\Delta C_1 + \Delta C_2)/(1 - (\Delta C_2/\Delta C_1)^2)$ and probability of release $p = (1 - \Delta C_2/\Delta C_1)$ (see [26]) associated to T- and L-type channels. The RCCs contained also R-type channels that were common to both groups of cells (adapted from [8] and [25])
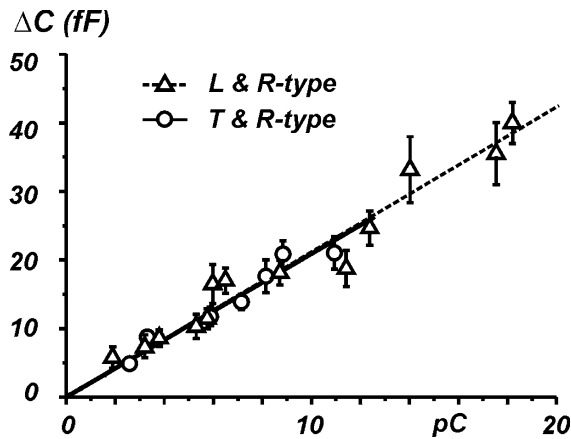
ΔC (fF)

Fig. 5 T- and L-type channels are coupled to exocytosis with the same efficacy for Ca^{2+}. ΔC increases vs the corresponding quantity of charge for cAMP-treated RCCs expressing T- and R-type channels and incubated in a medium containing ω-toxins and nifedipine (*empty circles, solid line*) compared to ΔC increases induced by L- and R-type channels expressed in control RCCs treated with ω-toxins (*empty triangles, dashed line*). The linear regressions have almost equal slope: 2.0±0.1 fF/pC (T- and R-type) and 2.1±0.1 fF/pC (L- and R-type) (adapted from [8] and [25])

of large dense-core vesicles) that decreases drastically with time (Fig. 3). ΔC saturates with prolonged depolarizations because of the approaching complete mobilization of the immediately releasable pool (IRP) of vesicles; another reason is the fast inactivation of T-type currents, which 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 a maximal rate of release of 580 fF/s [8] and 680 fF/s [32] for the same cell preparation. As the maximal rate of release is proportional to the channel density, the lower value estimated for T-type channels may just be the consequence of their lower density of expression with respect to HVA channels. A high initial rate of release associated to T-type channels can also be estimated in MPC9/3L cells (250 vesicles/s) if assuming that a membrane capacitance change of 1 fF in these cells would correspond to the release of five vesicles with mean diameter between 50 and 100 nm.

A second important observation is that the size of the IRP and the probability of release (p) estimated by double-pulse protocols (Fig. 4a) are comparable to those associated to HVA channels (Fig. 4b). 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. It is interesting to notice that the estimate of IRP and p using the double-pulse protocol requires unusually long interpulse intervals (1.5 s) to recover T-type channels from inactivation so as to have comparable Ca^{2+} entry during the two pulses. This contrasts with the basic principle of the method, which imposes short interpulse intervals to avoid vesicle refilling during pulses [26]. In the case of T-type channels, however, this causes a 15–20% overestimate of

the IRP that is partially compensated by the 5–10% decrease of Ca^{2+} currents during the second pulse.

Finally, the Ca^{2+} dependence of exocytosis evaluated by plotting the ΔCs vs the corresponding quantity of Ca^{2+} charges (~2 pF/pC) is roughly linear and is similar to that estimated for the L-type channels in the same preparation (Fig. 5). This finding has two main implications. First, T-type and L-type channels couple with the same Ca^{2+} efficacy to exocytosis. Because the same degree of linearity present between Ca^{2+} charges and exocytosis exists also for the other Ca^{2+} channels expressed in RCCs (R, N, and P/Q), this suggests that in RCCs, there is no preferential coupling between the channels belonging to the Ca_v2 family and release sites. Second, a high probability of release and a linear Ca^{2+} dependence similar to that of HVA channels imply that T-type channels contribute to the release of catecholamine at low voltages with the same efficacy that HVA channels contribute to the secretion at high voltages. In addition, considering that T-type channels deactivate at lower rates and that action potentials in RCCs have a prominent and relatively slow repolarization phase, which favor Ca^{2+} entry through LVA channels, it is likely that the contribution of T-type channels may be determinant during spontaneous action potentials firing near resting (2–3 Hz) or during trains of 15–20 Hz. Taken together, all these findings indicate that T-type channels are effectively coupled to exocytosis in RCCs and that they contribute to secretion with the same efficacy of HVA channels normally expressed in RCCs.

T-type channels do not couple to secretion in immature chromaffin cells

T-type channels do not produce fast secretion coupling at all stages of chromaffin cell development. In embryonic RCCs, LVA channels are expressed in half of the cells but Ca^{2+} currents through these channels elicited by 160 ms pulse depolarizations are not able to evoke sizable secretion [7]. On the contrary, secretion measured by capacitance changes and amperometric detection is evident in the other half of cells expressing HVA channels, thus suggesting that vesicles and the overall secretory apparatus is effectively coupled to voltage-gated Ca^{2+} channels. A possible explanation for the absence of T-type channel secretion coupling is that in immature RCCs, LVA channels are not sufficiently localized to secretory vesicles to induce secretion even when extracellular Ca^{2+} is raised to produce Ca^{2+} entry in the order of 60 pC. A reason for this could be due to the lower density of T-type channel expression in immature vs mature RCCs (6.5 pA/pF at −10 mV in 10 mM Ba^{2+} vs 15 pA/pF at −10 mV in 10 mM Ca^{2+}, [49]), but it cannot be excluded the absence of critical factors associated to secretion or an

increased Ca^{2+} buffering capacity in immature RCCs that may prevent Ca^{2+} to reach the release sites when T-type channels are too far apart. This latter condition indeed plays a critical role in chromaffin cells due to the significant distance at which voltage-gated Ca^{2+} channels are usually located from release sites (~200 nm on average) [38]. The lack of secretion does not also seem to depend on the isoform of T-type channels expressed in immature RCCs. In fact, based on the fast inactivation of LVA currents in immature RCCs, it appears that the underlying channel is either the α_{1H} or the α_{1G} subunit, and both channel types are shown to be effectively coupled to rapid secretion in mature RCCs (α_{1H}) and MPC9/3L cells (α_{1G}).

Are α_{1H} T-type channels "stress-induced" channels?

Overexpression of α_{1H} T-type channels during exposure to cAMP [25], β_1-adrenergic stimulation [49], and chronic hypoxia [9, 19], and their effective coupling to catecholamine secretion suggest that T-type channels of chromaffin cells may be conditional in the regulation of adrenal medulla response to stress conditions. Chromaffin cells play a key role in the response to stress and are an excellent model for studying the effects of stress on gene expression. Repeated stress like immobilization periods of 2 h and hypoxia are shown to produce an immediate release of catecholamines (adrenaline, noradrenaline, and dopamine) in the adrenal medulla. This is followed by a fast upregulation of catecholamine-synthesizing enzymes (TH, DBH, and PNMT) and an increased expression of a number of genes encoding the transcription factors for TH, DBH, and PNMT mRNA synthesis plus other genes related to kinases/phosphatase signaling and neurosecretion [56].

cAMP plays a key role in the regulation of these processes. The cAMP-response element binding protein is involved in the rapid induction of TH and DBH transcription in chromaffin cells [56], and cAMP triggers the rise of the PNMT-promoter-driven gene activity in PC12 cells in hypoxic conditions [62]. Thus, exposure to cAMP, chronic hypoxia, and β_1-adrenergic stimulations (which raise the level of cAMP) appear to be the suitable factors for mimicking prolonged stress conditions in vitro. We found that all these factors induce a common response in RCCs: a marked recruitment of α_{1H} T-type channels through cAMP-receptor proteins (Epac) [49]. This lowers the threshold of cell excitability and increases the quantity of catecholamine secreted near resting potentials [25]. Under these conditions, an upregulation of T-type channels effectively coupled to catecholamine release enhances the functional role of these channels during conditions mimicking stress-induced sympathetic stimulations ("fight-or-flight" response). T-type channels appear therefore as key elements

of the positive feedback originating from the release of adrenaline and noradrenaline [63], the autocrine activation of β_1-adrenoreceptors [15] and the increased levels of cAMP. These latters activate Epac, transcription factors, gene expression, protein synthesis and trigger important adaptive responses to stressful conditions. An effective availability of T-type channels at potentials near resting (-50 to -60 mV) is ensured by the existence of a significant "window" current with peak amplitude at -45 mV in 10 mM Ca^{2+} [49] (see also [18]) and by the prolonged repolarization to -60 and -70 mV between action potentials occurring at 2–3 spikes/s.

Conclusions

Evidence is accumulating on a key role of T-type channels in transmitter release. Recent works have brought indisputable evidence that T-type channels are capable of controlling hormone and neurotransmitters release in association with the exocytosis of large dense-core and synaptic vesicles. The T-type channel–secretion coupling is not as tight as that reported for N- and P/Q-type channels of central neurons, which allows high rates of vesicle release and rapid synaptic responses. Nevertheless, T-type channel–secretion coupling is sufficiently fast to drive the quick fusion of vesicles ready for release during low-threshold Ca^{2+} fluxes. T-type channels appear to be effectively coupled to rapid vesicle depletion and are possibly involved in the regulation of Ca^{2+}-dependent vesicle recycling, which is an important key process supporting synaptic activity during high-frequency stimulation.

This review aims at furnishing convincing proofs that T-type channels possess all the prerequisites for controlling fast transmitter release in a number of central synapses and secretory cells. It is likely that these observations will soon be extended to other neuronal preparations in which the contribution of LVA channels has been overlooked or not properly investigated. This search would certainly improve if increased attention will be focused on the contribution of T-type channels to presynaptic Ca^{2+} currents and if high-affinity selective blockers for LVA channels would become soon available.

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