

Fast exocytosis mediated by T- and L-type channels in chromaffin cells: distinct voltage-dependence but similar Ca^{2+} -dependence

V. Carabelli · A. Marcantoni · V. Comunanza · E. Carbone

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Abstract Expression, spatial distribution and specific roles of different Ca^{2+} channels in stimulus–secretion coupling of chromaffin cells are intriguing issues still open to discussion. Most of the evidence supports a role of high-voltage activated (HVA) Ca^{2+} channels (L-, N-, P/Q- and R-types) in the control of exocytosis: some suggesting a preferential coupling of specific Ca^{2+} channel subunits with the secretory apparatus, others favoring the idea of a contribution to secretion proportional to the expression density and gating properties of Ca^{2+} channels. In this work we review recent findings and bring new evidence in favor of the hypothesis that also the LVA (low-voltage-activated, T-type) Ca^{2+} channels effectively control fast exocytosis near resting potential in adrenal chromaffin cells of adult rats. T-type channels recruited after long-term treatments with pCPT-cAMP (or chronic hypoxia) are shown to control exocytosis with the same efficacy of L-type channels, which are the dominant Ca^{2+} channel types expressed in rodent chromaffin cells. A rigorous comparison of T- and L-type channel properties shows that, although operating at different potentials and with different voltage-sensitivity, the two channels possess otherwise similar Ca^{2+} -dependence of exocytosis, size and kinetics of depletion of the immediately releasable pool and mobilize vesicles of the same quantal size. Thus, T- and L-type channels are coupled with the same Ca^{2+} -efficiency to the secretory apparatus and deplete the same number of vesicles ready for release.

The major difference of the secretory signals controlled by the two channels appear to be the voltage range of operation, suggesting the idea that stressful conditions (hypoxia and persistent β -adrenergic stimulation) can lower the threshold of cell excitability by recruiting new Ca^{2+} channels and activate an additional source of catecholamine secretion.

Introduction

N- and P/Q-type Ca^{2+} channels control neurotransmitter release in neurons, even though growing evidence support a role also for L- and T-type channels (Carbone et al. 2006b). A widely used model for studying neurosecretion and its coupling with Ca^{2+} channel activation are the chromaffin cells of the adrenal gland (Unsicker et al. 2005), which derive from the neural crest and share most of the proteins involved in vesicle fusion at presynaptic terminals. In these regions, strict colocalization of Ca^{2+} channels and vesicles is required (active zones) to achieve fast synchronous synaptic activity, which is usually not critical in neuroendocrine cells (Kits and Mansvelder 2000).

In vivo splanchnic nerve stimulation evokes a sequence of events that includes rhythmic chromaffin cell depolarization, Ca^{2+} -entry through voltage-gated Ca^{2+} channels, fusion of large dense core granules with the chromaffin cell membrane and subsequent release of catecholamines and other neurotransmitters into blood circulation. This slower exocytotic process is primarily controlled by Ca^{2+} influx through multiple isoforms of high-voltage activated Ca^{2+} channels, which contribute to secretion proportionally to their density of expression and gating properties, with no specific

V. Carabelli (✉) · A. Marcantoni · V. Comunanza · E. Carbone
Department of Neuroscience, Centre of Excellence NIS, CNISM UdR, Corso Raffaello 30, 10125 Turin, Italy
e-mail: valentina.carabelli@unito.it

requirements of Ca^{2+} channels colocalization to the release sites (Kim et al. 1995; Engisch and Nowycky 1996; Klingauf and Neher 1997; Lukyanetz and Neher 1999; Ulate et al. 2000; Carabelli et al. 2003; Chan et al. 2005; Giacippoli et al. 2006; Polo-Parada et al. 2006). Alternatively, other groups suggest a preferential role of some channel subtype in close proximity to the secretory sites (Lopez et al. 1994; Artalejo et al. 1994; Lara et al. 1998; O'Farrel and Marley 2000; Albillos et al. 2000) and a further role of mitochondria in sequestering Ca^{2+} entering through Ca^{2+} channels (Ales et al. 2005). In addition to this, the efficiency of stimulus–secretion coupling can be altered in many ways, some relevant examples are: (1) PKC activation, which enhances exocytosis (Gillis et al. 1996), (2) rapid and sustained increase of intracellular cAMP, which potentiates L-type channel densities and secretion at different proportions (Carabelli et al. 2003), (3) transdifferentiation of chromaffin cells with astrocyte-conditioned medium, which promotes the acquisition of a neuronal phenotype and accelerates the exocytotic kinetics, by a better coupling between secretory vesicles and Ca^{2+} channels (Ardiles et al. 2006) and (4) β -adrenergic stimulation, which enhances the stimulus–secretion coupling of L-type Ca^{2+} channels by repeated action potential-like stimulations (Polo-Parada et al. 2006).

Interestingly, T-type channels are the only Ca^{2+} channel types not normally expressed in chromaffin cells of adult animals but can be recruited by long lasting pCPT-cAMP incubation (Novara et al. 2004) or chronic exposures to hypoxia (Carbone et al. 2006a; Del Toro et al. 2003). The possibility of recruiting a sufficient density of T-type channels by either long-term cAMP incubations or overnight exposure to low PO_2 offers a unique opportunity for testing how a newly recruited Ca^{2+} channel population is localized at the release zone and contribute to neurotransmitter release. Of particular relevance is whether T-type channels are capable of coupling to secretion and preserve their characteristic low-threshold of activation. In addition, it is interesting to assay how close the secretory events follow the voltage-dependence of T-type channels and how T-type channels may coexist with the other voltage-gated Ca^{2+} channels normally expressed in rodent chromaffin cells, in particular with the L-type channels which are the dominant Ca^{2+} channel subtypes controlling secretion in these cells (see Garcia et al. 2006). Besides being the most densely expressed Ca^{2+} channels in rodent chromaffin cells, L-type channels possess other important features: (1) control 30% of catecholamine release in in-vivo perfused rat adrenal glands while P/Q and N-type channels contribute marginally (Akiyama et al. 2004), (2)

can be up- and down-modulated by β -adrenergic auto-receptors (Hernandez-Guijo et al. 1999; Cesetti et al. 2003) and affected by phosphodiesterase type-4 activity (Marcantoni et al. 2006), (3) contribute to a low-threshold “pace-maker” current that sets the action potentials autorythmicity at rest (Carbone et al. 2006c).

Here, by reanalyzing and summing-up the results of two recent papers (Carabelli et al. 2003; Giacippoli et al. 2006), we show that in chromaffin cells the two channels (T- and L-type) although lacking a “synprint” region, appear equally effective in controlling neurotransmitter release despite their different voltage-dependence, activation–inactivation gating and degree of expression. A rigorous analysis of the biophysical parameters associated with the excitation–secretion coupling of the two channels shows close similarities. In fact, low-voltage activated T-type channels possess the same Ca^{2+} -dependence of secretion, probability of release and mobilize a pool of readily releasable vesicles comparable in number and unitary size to that mobilized by L-type channels. This implies that T-type channels form an effective Ca^{2+} -signaling pathway suitable for both lowering the threshold of action potential firing and triggering low-threshold exocytosis. A corollary of our study is that Ca^{2+} channels having different voltage-range of activation and gating mechanisms preserve intact their gating features even if directly involved in large dense core granules exocytosis. This may also suggest that the secretory apparatus is incapable of altering Ca^{2+} channel gating, most likely because secretory vesicles are sufficiently apart (200–300 nm) to sense Ca^{2+} elevation without directly interacting with the channel structure.

Methods

Isolation and culture of rat chromaffin cells

Rat chromaffin cells were obtained from adrenal glands as described elsewhere (Giacippoli et al. 2006). Adult animals (200–300 g) were sacrificed in accordance with the guidelines established by the National Council on Animal Care. Isolated chromaffin cells were plated on plastic dishes pretreated with poly-L-ornithine (1 mg/ml) and laminin (5 $\mu\text{g}/\text{ml}$ in L-15 carbonate), cultured at 37°C in water-saturated atmosphere with 5% CO_2 and used for electrophysiological recordings within 6 days after plating. The culture medium was DMEM supplemented with antibiotics (50,000 units/l penicillin, 50 mg/l streptomycin, 125 mg/l gentamycin) and 15% fetal calf serum (Carabelli et al. 2003). To recruit T-type channels 200 μM pCPT-cAMP

was added to the serum-free medium 24 h after plating (Novara et al. 2004).

Electrophysiological recordings

An EPC-9 patch-clamp amplifier (Heka Electronic; Lambrecht, Germany) was used for simultaneous recording of Ca^{2+} currents and secretion determined through membrane capacitance increases induced by depolarizations. Patch-clamp pipettes were obtained from Kimax borosilicate glass (Witz Scientific, Holland, OH, USA) and then fire-polished; the final series resistance was 1.2/1.8 M Ω . Ca^{2+} currents were sampled at 10 kHz and filtered at 2 kHz. Experiments were performed in the perforated-patch configuration, at room temperature (22–24°C), using amphotericin (Sigma, St Louis, MO, USA). Recordings started only after the access resistance was below 20 M Ω , and this occurred within some minutes after the seal. Membrane capacitance increases (ΔC) following depolarizations were measured by applying a sinusoidal wave function (± 25 mV amplitude, 1 kHz frequency); the increment was calculated by measuring the mean capacitance after the pulse (averaged over 400 ms) respect to a reference value before the pulse. Data are given as mean \pm SEM for n = number of cells. Statistical significance was calculated using unpaired Student's t test and P values < 0.05 considered significant.

Solutions

The perforated-patch configuration was obtained using amphotericin B, dissolved in dimethyl sulfoxide (50 mg/ml) and stored at -20°C . The final concentration was 50–100 $\mu\text{g/ml}$. The pipette filling solution contained (mM): 135 CsMeSO₃, 8 NaCl, 2 MgCl₂, 20 HEPES (pH 7.3 with CsOH). The extracellular solutions for measuring L- and T-type channels were, respectively (mM): 135 NaCl, 2.8 KCl, 10 CaCl₂, 2 MgCl₂, 20 glucose, 10 HEPES and 145 TEACl, 5 CaCl₂, 10 glucose, 10 HEPES. The two ionic conditions determine an approximately 4 mV positive shift in the liquid junction potential and an 8 mV negative shift of membrane voltage due to the lower screening of membrane surface charges when measuring T-type currents in 5 mM Ca^{2+} and 145 TEA⁺, with respect to L-type currents measured in 10 mM Ca^{2+} and 135 Na⁺. This implies that the voltage-dependent parameters of T-type channels are biased by a 4 mV negative shift with respect to L-type channels. Notice that under similar recording conditions the resting free Ca^{2+} concentration in rat chromaffin cells ranges between 500 and 700 nM (Micheletti et al. 2005).

Results

T-type and L-type channels induce fast secretion with sharply different threshold of activation and voltage-dependence

In rat chromaffin cells, the threshold of detection of charge (Q) entering during 100 ms depolarizations and depolarization-evoked capacitance increase (ΔC) are shifted by approximately 30 mV toward negative potentials when T-type channels are available after long-term pCPT-cAMP treatments (Fig. 1c, d). Representative traces of low-threshold T-types and high-threshold L-type currents and capacitance increases at -30 and $+10$ mV are shown in Fig. 1a, b. In both cases, cells were pre-treated with ω -CTx GVIA (3.2 μM) and ω -Aga IVA (2 μM) to block the contribution of N and P/Q types Ca^{2+} channels. For better isolating T-type channels, nifedipine (1 μM) was added to the external solution (Fig. 1a). These pharmacological conditions ensure the absence of L, N and P/Q-type currents while measuring cAMP-recruited T-type currents, but do not prevent the presence of R-type channels that partially contribute to the total current. The same is true when measuring L-type currents after ω -toxins treatment. Therefore, the present comparison of T- and L-type current recordings is biased by the presence of R-type currents that, on average, are expected to contribute equally in both conditions. It is also worth noticing that recordings of L-type currents are biased by the presence of a fast inactivating Na⁺ current that does not contribute to Ca^{2+} -dependent exocytosis. The conditioning prepulse to -40 mV in Fig. 1b was applied to block T-type channels that could be available in normal conditions.

By comparing the voltage-dependence of the Ca^{2+} charges entering the cells during depolarization ($Q(V)$) and the size of the capacitance increases induced by Ca^{2+} influx, $\Delta C(V)$, there are two important features to underline. There is an impressive correlation between the voltage-dependence of the two parameters for the same channel and a sharply different voltage-dependence between the two channels. In the case of T-type, the $Q(V)$ and $\Delta C(V)$ peak between $+5$ and $+10$ mV and have comparable half-maximal values $V_{1/2}$ (-32 and -34 mV) and steepnesses k (an e-fold change for a 10 and 13 mV potential variation). In the case of L-type channels the voltage-dependence of $Q(V)$ and $\Delta C(V)$ is nearly two times steeper than T-types ($k = 5$ and 6 mV) and $V_{1/2}$ is about 20 mV more positive (-13 and -10 mV). These results suggest that Ca^{2+} -entry and secretion induced by T- and L-type channels follow rather strictly the voltage-sensitivity of the two channel

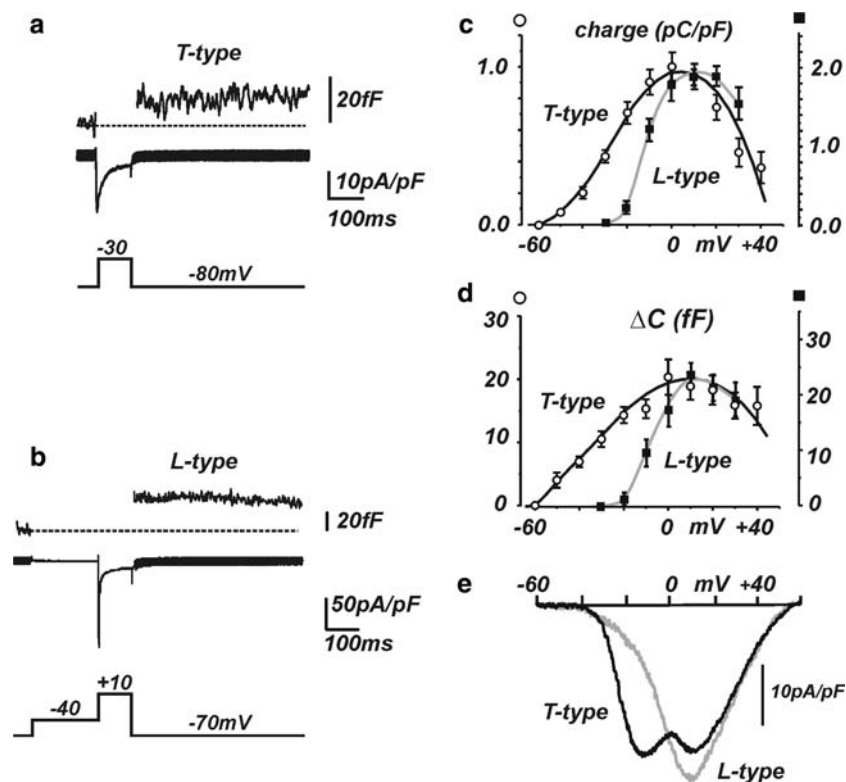


Fig. 1 Depolarization-evoked secretion induced by T-type and L-type Ca^{2+} channel activation. Cells were pretreated with ω -Ctx GVIA (3.2 μM) and with ω -Aga IVA (2 μM) to block N- and P/Q-type channels. **a** T-type currents were isolated by adding nifedipine 1 μM and evoked by test depolarization at -30 mV. The upper trace indicates the capacitance increment (ΔC) caused by the corresponding pulse depolarization. **b** L-type currents at $+10$ mV and related depolarization-evoked exocytosis. The conditioning prepulse (-40 mV) was applied in order to inactivate residual N- and P/Q-type currents still available after toxin incubation. **c, d** Mean quantity of charge and capacitance increment at different

types. Notice that a good correlation exists also between the $V_{1/2}$ of the I–V curves for the two channels and the $V_{1/2}$ of $Q(V)$ and $\Delta\text{C}(V)$ (Fig. 1e). L-type currents have half-maximal activation around -8 mV (Cesetti et al. 2003) and T-type currents have $V_{1/2}$ at -26 mV.

T-type and L-type channels are similarly coupled to the secretory sites

Having established that T-type and L-type channels give rise to secretory activities in well separated voltage ranges ($\Delta V \sim 20$ mV), the next important issue is to establish how T-type and L-type channels are coupled to secretory sites. To do this, one needs to determine the size of the immediately releasable pool (IRP), the rate of vesicle release, the probability of release and the Ca^{2+} -dependence of secretion when the two channels are selectively activated (Carabelli et al. 2003;

Giancippoli et al. 2006). According to the model described by Voets (2000), secretory granules in chromaffin cells are distributed into three different pools, with their own size, kinetics of release and fusion competence. Among them, the IRP is supposed to be constituted by $\sim 10\%$ of vesicles “morphologically docked” near the membrane, which can experience a higher concentration of Ca^{2+} and can be released after brief depolarizing pulses with an extremely fast kinetics. Size and kinetics of depletion of the IRP can be accurately quantified by increasing the intracellular Ca^{2+} concentration and measuring the relative capacitance increment (Henkel and Almers 1996; Kits and Mansvelter 2000).

There are two different methods to quantify the parameters characterizing vesicle secretion. One uses a double-pulse protocol (Gillis et al. 1996), in which two pulses, separated by a short time interval (50–100 ms), are applied (Fig. 2a, b). The pulses produce two ΔC

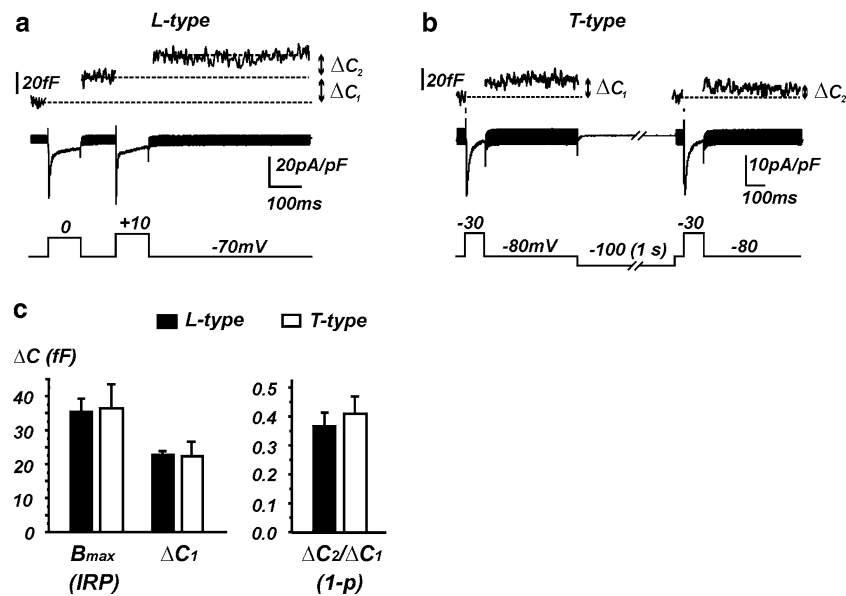


Fig. 2 Evaluation of the IRP size and probability of release. **a, b** The double pulse protocol is applied for L- and T-type channels, respectively. The longer hyperpolarizing interpulse in **b** (1 s, -100 mV) allows recruiting T-type channels from inactivation. **c** The maximum size of the IRP was estimated from the sum ($\Delta C_1 + \Delta C_2$) and the ratio ($\Delta C_2/\Delta C_1$) of the consecutive capacitance increases, according to the following relationship:

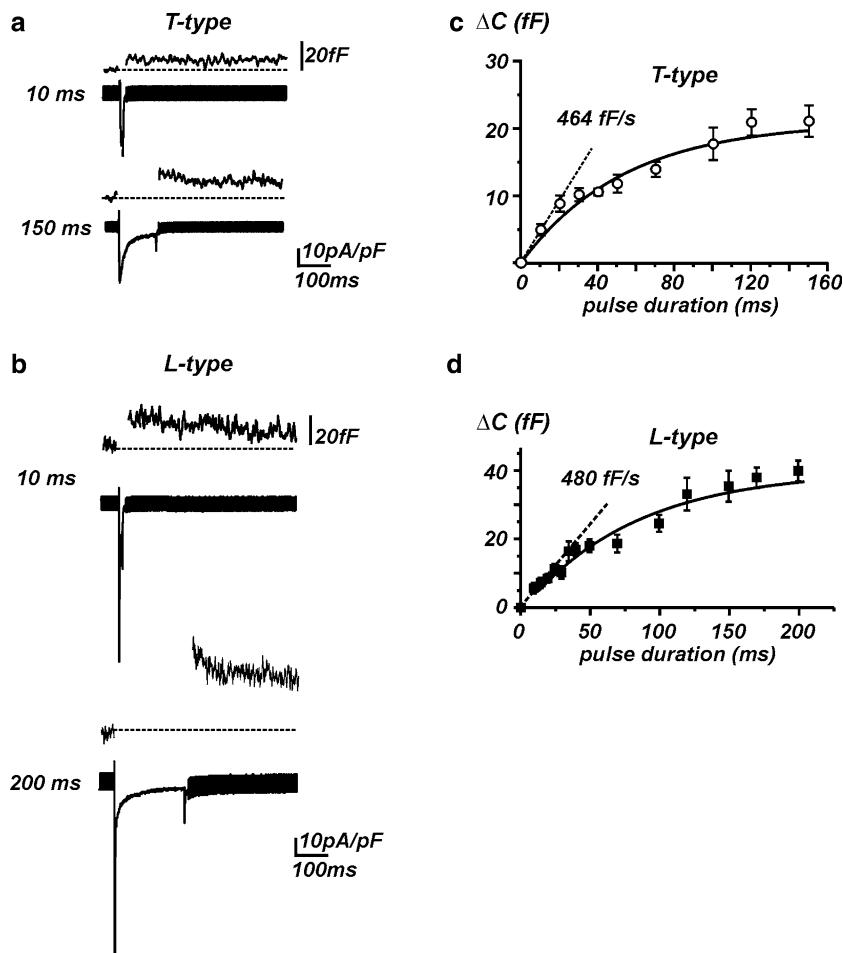
$B_{max} = (\Delta C_1 + \Delta C_2)/(1 - (\Delta C_2/\Delta C_1)^2)$, whereas ΔC_1 sets the lower limit of the IRP (Gillis et al. 1996). Probability of release ($P = 1 - \Delta C_2/\Delta C_1$) is high in both conditions, suggesting tight functional coupling between channels and secretory sites. Mean values were obtained from $n = 10$ cells (L-type) and $n = 12$ cells (T-type). Adapted from Carabelli et al. (2003) and Giancippoli et al. (2006)

increases (ΔC_1 and ΔC_2) and are requested to carry the same quantity of Ca^{2+} charge. In this way, from the amplitude of ΔC_2 one can estimate the percentage of vesicle depletion that occurs during the first pulse (ΔC_1) and with simple calculation one can determine the size of IRP and the probability of release (P). This method has two drawbacks: (1) it underestimates the size of the IRP if partial refilling of the pool occurs during the interpulse, (2) in the case of T-type channels, short interpulses are insufficient to recover the channels from fast inactivation and thus the double pulse should be separated by long interpulse intervals (1–1.5 s). Since the time of vesicle replenishment is in the order of 10–15 s in rat chromaffin cells, the error due to partial refilling of the pool appears not to be determinant for the estimate of IRP and P . According to this protocol it results that, independently of each other, both channels mobilize an IRP of nearly the same size that varies between 23 fF (ΔC_1) and 37 fF (IRP), corresponding to a pool of ~37 vesicles (Fig. 2c). Of relevance is also the observation that probability of release is equally high ($P \sim 0.6$) regardless of the type of functioning channels, suggesting that the overall release apparatus controlled by the two channels has close similarities.

The second method evaluates the IRP size by measuring the ΔC increases during pulses of increasing length until a plateau value is reached (Horrigan and

Bookman 1994). By definition, this value furnishes the total number of vesicles that can be released during maximal Ca^{2+} entry through Ca^{2+} channels (Fig. 3a, b). The method allows also determining the rate of vesicle release during brief pulses (10 ms) whose duration is comparable to the action potential durations (5 ms) and thus allows determining how effective is the coupling of Ca^{2+} channels to secretion near physiological conditions. Fig. 3c and d show that in both cases secretion increases exponentially and reaches plateau levels for pulse of 150–200 ms. For the L-type channel the asymptotic value is 40 fF, in good agreement with the IRP value obtained with the double-pulse method (37 fF) and with previous data on rat and mouse chromaffin cells (Horrigan and Bookman 1994; Voets et al. 1999). In contrast to this, for the T-type channel there is a clear discrepancy between the values estimated with the two methods (21 vs. 35 fF), but there is also a clear explanation for this. T-type channels are fast inactivating and thus the increase of Ca^{2+} entry during pulses longer than 100 ms is drastically reduced. In this case, the Horrigan and Bookman’s method largely underestimates the IRP size. However, it is interesting to notice that during very brief pulses the two channel types induce a comparably rapid release of vesicles that can be estimated from the slope of the $\Delta C(t)$ curve at time zero (464 and 480 fF/s; dashed lines in Fig. 3c, d). This implies that both channels are very effective in

Fig. 3 Evaluation of the IRP by saturating Ca^{2+} influx through T- and L-type Ca^{2+} channels. The IRP size has been evaluated by applying depolarizing pulses of increasing lengths, from 10 to 150 ms for T-type (a), and to 200 ms for the L-type channels (b), and measuring the corresponding ΔC . c, d mean ΔC increases are plotted versus the pulse length for $n = 20$ (T-type) and $n = 19$ cells (L-type), in order to measure the IRP size (from the plateau value) and the initial kinetics of exocytosis (from the slope of the exponential fit). The corresponding values are specified in the text. Adapted from Carabelli et al. (2003) and Giacippoli et al. (2006)



controlling vesicle release during time intervals comparable to the mean duration of action potential overshoot.

The best evidence that T- and L-type channels control equally well the secretion in rat chromaffin cells comes from the results of Fig. 4 where the values of ΔC from Fig. 3c and d are plotted versus the quantity of

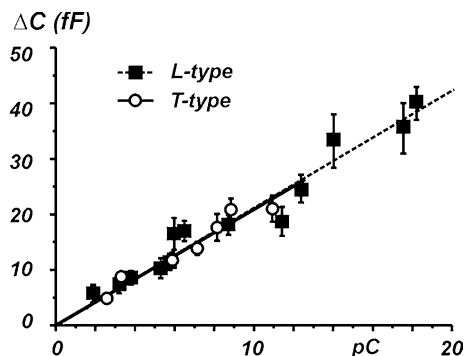


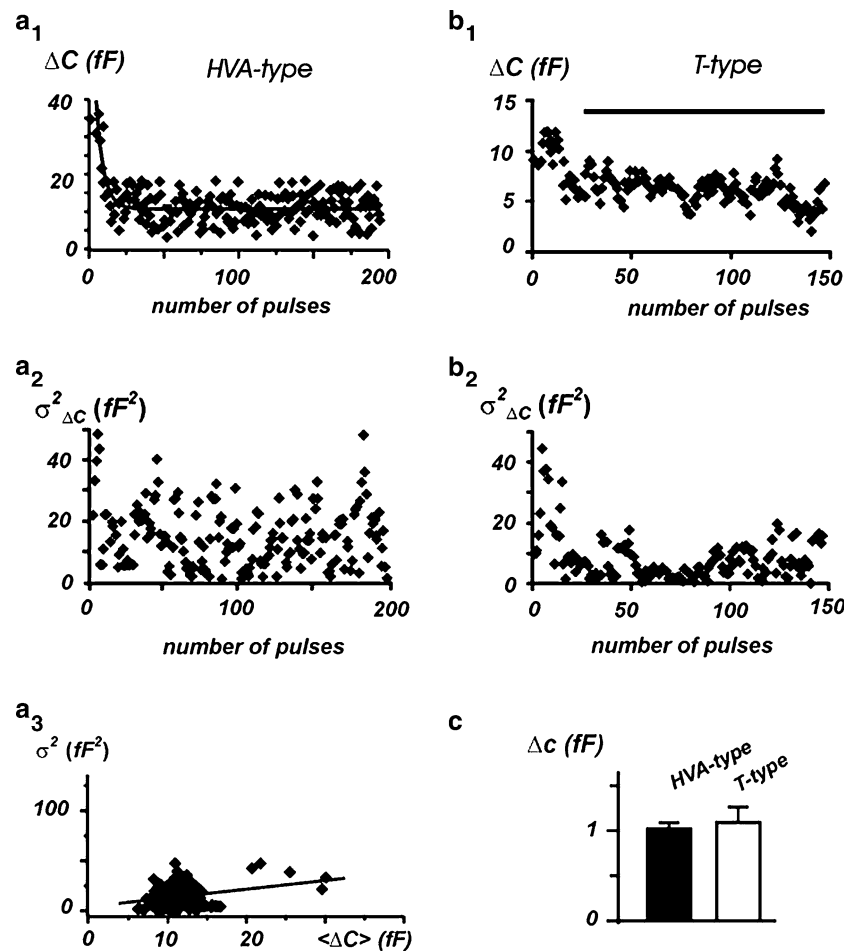
Fig. 4 Ca^{2+} -dependence of exocytosis. Ca^{2+} -dependence of exocytosis is obtained by plotting the mean ΔC increases of Fig. 3c, d versus the corresponding quantity of charge and measuring the slope of the regression line. These data show that T- and L-type channels have very similar Ca^{2+} -dependence (efficiency). Adapted from Carabelli et al. (2003) and Giacippoli et al. (2006)

Ca^{2+} entering during a pulse (integral of the Ca^{2+} current). In this way the inconvenience of Ca^{2+} channel inactivation is overcome and secretion is compared directly to the quantity of Ca^{2+} entering the cell. It is evident that Ca^{2+} -dependence of exocytosis is roughly linear for both channels and have similar slope (2.0 and 2.1 fF/pC).

T-type and L-type channels mobilize vesicles of the same unitary size

As shown in Fig. 5c, the vesicles mobilized by T- and L-type channels have a comparable mean unitary size (~ 1 fF). This suggests that localization of T-type channels occurs near vesicles of similar size to that mobilized by L-type channels. In other words, incorporation of new functioning T-type channels in the plasma membrane does not substantially change the membrane matrix involved in vesicle priming and vesicle docking. We can thus exclude that expression of newly functioning T-type channels does not produce vesicles doubling as in the case of brief, acute cAMP-treatment (Carabelli et al. 2003). A confirmation of this, however,

Fig. 5 Estimate of the size of single exocytic events. **a₁**, **b₁** Consecutive ΔC increases associated with brief depolarizations at +10 mV and –20 mV, for HVA and T-type channels. In **a₁** values are fitted by an exponential decreasing function, representing the secretory rundown due to vesicle depletion. **a₂** Trend-corrected variances evaluated by a moving-bin analysis of four consecutive ΔC values of panel **a₁**. **b₂** Variances evaluated by moving-bin analysis with four ΔC values of panel **b₁**. **a₃** Positive correlation of trend-corrected sample variances versus the corresponding mean ΔC values of **a₁**. **c** Mean size of single exocytic event for T-type ($n = 10$ cells) and high-voltage activated ($n = 9$ cells) Ca^{2+} channels calculated with the equations given in the text. Adapted from Carabelli et al. (2003) and Giacippoli et al. (2006)



requires high-resolution amperometric measurements that furnish a direct estimate of the amount of catecholamines released after the fusion of single granules (Machado et al. 2001).

Since chromaffin granules cannot be detected as single fusion events due to the low signal-to-noise ratio of the recording apparatus, the single quantal size can be estimated only by non-stationary fluctuation analysis of repeated ΔC events, as described by Moser and Neher (1997) (Fig. 5a, b). Following this method, the unitary size of secretory events associated with HVA channels (Δc) is derived indirectly from the fluctuations of the secretory response during a train of short depolarizations (20 ms) at +10 mV applied at a frequency of 0.3 Hz, for about 10 min. As shown in Fig. 5a₁, variations among ΔC increases are larger at the beginning of the train and decay with time due to vesicle depletion during pulses repeated every 3 s. In order to compensate for the decline in the secretory response, the variances at different times ($\sigma^2_{\Delta C}$; Fig. 5a₂) are calculated by moving-bin analysis of four consecutive ΔC increases after subtracting a fit (solid line) to the mean ΔC values. Linear regression of variances versus the

corresponding means gives a slope of 0.9 fF (Fig. 5a₃), which represents the estimated capacitive increment of a single granule (Δc), according to the equation: $\sigma^2_{\Delta C} = \Delta c \langle \Delta C \rangle$.

To estimate the single exocytic event associated with T-type channels, the protocol is slightly modified, due to the small capacitance increases caused by brief depolarization at low potentials. Pulses at –20 mV are applied with the same frequency; ΔC declines rapidly within the first 30 depolarizations and then reaches a steady-state value, as indicated by the continuous line in Fig. 5b₁. In this time interval, the variance of the stationary fluctuations is used to calculate Δc , according to the equation: $\Delta c (1-P) = \sigma^2_{\Delta C} / \langle \Delta C \rangle$, with P indicating the release probability per vesicles. In this way, the estimated Δc associated with T-type channels is on average 1 fF, which is very similar to that of HVA channels (Fig. 5c).

Discussion

Cav1 L-type channels represent the major Ca^{2+} current component in rat chromaffin cells, where both α_{1C}

(Cav1.2) and α_{1D} (Cav1.3) subunits are expressed (Baldelli et al. 2004). Besides being highly expressed, L-type channels possess a number of properties that make them particularly important in the regulation of catecholamine release in rodent chromaffin cells. First, L-type channels are shown to markedly contribute to secretion in voltage-clamped rat chromaffin cells (Kim et al. 1995) where exists an impressive linearity between Ca^{2+} current densities and membrane capacitance changes (Carabelli et al. 2003). This suggests that when activated with voltage-clamp protocols, all HVA channels contribute proportionally to secretion and L-type channels dominate mainly because they are more densely expressed. Second, L-type channels are shown to effectively control catecholamine release in in-vivo micro-dialyzed adrenal glands of anesthetized animals, while P/Q and N-type channels contribute marginally (Akiyama et al. 2004). Third, L-type channels can be up- and down-modulated by secreted products through the activation of β -adrenergic autoreceptors (Hernandez-Guijo et al. 1999; Cesetti et al. 2003) and play a regulatory function in the depolarization-evoked exocytosis in chromaffin cells (Carabelli et al. 2003; Nagy et al. 2004). Fourth, recent data suggest an additional role for L-type channels, which can act as Ca^{2+} sensor proteins in neuroendocrine secretion (Lerner et al. 2006). The occupancy of L-type channel pore by an impermeable cation, such as La^{3+} , lead to a conformational change that is transmitted to the exocytotic machinery upstream of intracellular Ca^{2+} concentration. Fifth, fluorescence microscopy imaging using evanescent light shows that L-type channels form Ca^{2+} microdomains around large-dense core vesicles docked within 300 nm and are directly involved in the Ca^{2+} signaling leading to vesicle fusion and release in bovine chromaffin cells (Becherer et al. 2003).

At variance with this, functioning T-type channels are rarely observed in the chromaffin cells of adrenal medulla; despite they largely contribute to hormone release in the zona glomerulosa of the cortical adrenal gland (Schrier et al. 2001). T-type channels are reported to coexist in bovine chromaffin cells (Garcia-Palomero et al. 2000), in a restricted proportion of adult rat chromaffin cells (Hollins and Ikeda 1996) and in a subpopulation of developing immature rat chromaffin cells (Bournaud et al. 2001). In this latter case, T-type channels fail to support secretion, not because of insufficient Ca^{2+} influx, but presumably because Ca^{2+} entering could be rapidly buffered, limiting the raise of intracellular Ca^{2+} to threshold levels required for triggering exocytosis. Despite very weakly expressed, the α_{1H} (Cav3.2) T-type channel is selectively recruited by prolonged cAMP increase consequent to β_1 -adrenergic

stimulation (Novara et al. 2004; Giaccipoli et al. 2006) or following chronic hypoxia conditions (Del Toro et al. 2003; Carbone et al. 2006b). A rough estimate of Ca^{2+} current densities, single channel conductance and probability of release suggest (see Giaccipoli et al. 2006) that newly expressed α_{1H} channels increase the Ca^{2+} channel density by about 40% (from 6 to 10 channels/ μm^2), when functioning together with L-types (above -10 mV), and contribute to 4 channels/ μm^2 near resting potentials, thus lowering the threshold of both cell excitability (Novara et al. 2004) and catecholamine secretion (Giaccipoli et al. 2006).

The increased quantity of charge available at low voltages may represent an additional component of exocytosis obtainable under stressful conditions, when the body catecholamine supply from the adrenal gland needs to be potentiated. This extra Ca^{2+} charge is not preferentially coupled to the functioning of the secretory apparatus, since Ca^{2+} ions entering the cell through low-threshold channels have the same efficacy in triggering exocytosis as high-voltage activated types. Indeed, the cAMP-recruited T-type channels rapidly deplete a small subset of docked granules ready for release, whose size is comparable to the one previously described for rat and mouse chromaffin cells. It is also worth pointing out that short- and long-term exposures of rat chromaffin cell to permeable pCPT-cAMP have remarkably different effects on cell excitability and secretion. A sudden increase of cAMP (30 min incubation, 1 mM) potentiates Ca^{2+} influx through L-type channels but also alters the Ca^{2+} -dependence of exocytosis, doubles the unitary secretory event and the IRP size. On the contrary, prolonged exposures to cAMP, as described here (2–3 days, 200 μM), selectively recruit T-type channels that are normally absent and for this reason add a low-threshold component of the secretory process.

A final evidence related to a direct involvement of T- and L-type channels in supporting rapid exocytosis, comes from measurements using a recently established mouse pheochromocytoma cell line (MPC 9/3L), which express most of the proteins involved in Ca^{2+} -dependent neurotransmitter release except voltage-gated Ca^{2+} channels. Transfection with either N-type (α_{1B} , β_{2a} , $\alpha_{2\delta}$; Cav2.2), L-type (α_{1C} , β_{2a} , $\alpha_{2\delta}$; Cav1.2) or T-type (α_{1G} , β_{2a} , $\alpha_{2\delta}$; Cav3.1) Ca^{2+} channel subunits, supported fast exocytosis with comparable efficiency and rapid increases in membrane capacitance (Harkins et al. 2003). The interesting issue in this case is that the Ca^{2+} -dependent efficiency of secretion is comparable for the three channel types regardless of the density of the expressed channel subunits (0.5–0.7 pF/pC), which is about a factor three lower than in rat chromaffin cells (Fig. 4). This indicates that the lack of the “synprint

region” on L- and T-type channels is not critical for the control of neurosecretion in neuroendocrine cells and that there is not preferential coupling of voltage-gated Ca^{2+} channels and secretory sites in these cells as well as in normal chromaffin cells.

As a final remark, the reported evidence that LVA T-type channels can contribute to exocytosis with the same efficiency of HVA Ca^{2+} channels in rat chromaffin cells open interesting possibilities for a role of these channels in the control of neurotransmitter release in other cell preparations. The role of T-type channels may become particularly critical during pathological and stressful conditions in which increased cAMP levels or reduced O_2 pressures may cause their up-regulation and coupling to neurotransmitter release near resting potential conditions. Further experiments are certainly required to confirm these observations and to obtain more information about T-type channels and secretory vesicle coupling.

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