

E. Carbone · V. Carabelli · T. Cesetti · P. Baldelli
J. M. Hernández-Guijo · L. Giusta

G-protein- and cAMP-dependent L-channel gating modulation: a manifold system to control calcium entry in neurosecretory cells

Received: 8 February 2001 / Revised: 6 April 2001 / Accepted: 9 April 2001 / Published online: 8 June 2001
© Springer-Verlag 2001

Abstract Voltage-gated Ca^{2+} channels are crucial to the control of Ca^{2+} entry in neurosecretory cells. In the chromaffin cells of adrenal medulla, paracrinally or autocrinally released neurotransmitters induce profound changes in Ca^{2+} channel gating and Ca^{2+} -dependent events controlling catecholamine secretion and cell activity. The generally held view of these processes is that neurotransmitter-induced modulation of the most widely expressed Ca^{2+} channels in these cells (N-, P/Q- and L-type) follows two distinct pathways: a direct membrane-delimited $G_{i/o}$ -protein-induced inhibition of N- and P/Q-type and a remote cAMP-mediated facilitation of L-channels. Both actions depend on voltage, although with remarkably different molecular and kinetic aspects. Recent findings, however, challenge this simple scheme and suggest that L-channels do not require strong pre-pulses to be recruited or facilitated. They are available during normal depolarizations and may be tonically inhibited by $G_{i/o}$ proteins activated by the released neurotransmitters. Like the N- and P/Q-channels, this autocrine modulation is localized to membrane microareas. Unlike N- and P/Q-channels, however, the inhibition of L-channels is largely independent of voltage and develops in parallel with cAMP-mediated potentiation of channel gating. As L-channels play a crucial role in the control of catecholamine release in chromaffin cells, the two opposite modulations mediated by $G_{i/o}$ proteins and cAMP may represent an effective way to broaden the dynamic range of Ca^{2+} signals controlling exocytosis. Here, we review the basic features of this novel L-type channel inhibition comparing it to the well-established forms of L-channel potentiation and voltage-dependent facilitation.

The material in this article was presented at the International Conference in Kiev, Ukraine, 10–12 September 1999, celebrating the 75th birthday of Professor Kostyuk.

E. Carbone (✉) · V. Carabelli · T. Cesetti · P. Baldelli
J.M. Hernández-Guijo · L. Giusta
Dipartimento di Neuroscienze, Unità di Ricerca I.N.F.M.,
Corso Raffaello 30, 10125 Torino, Italy
e-mail: emilio.carbone@unito.it
Tel.: +39-11-6707786, Fax: +39-11-6707708

Keywords Adrenal chromaffin cells · Calcium channels · cAMP/PKA-mediated phosphorylation · Opioidergic/purinergic/adrenergic-receptors · PTX-sensitive G proteins · Voltage-dependent facilitation

Introduction

The chromaffin cells of the adrenal medulla are one of the best examples of the neurosecretory system for studying hormone secretion and the correlations between Ca^{2+} channel function, Ca^{2+} entry and secretory events [7, 48, 99]. Chromaffin cells express L-, N-, P/Q-, and R-type Ca^{2+} channels that have diverse biophysical and molecular structure characteristics [1, 5, 46, 56, 61] and diverse modulatory responses to the neurotransmitters that are released during cell activity [2, 19, 29, 45, 57, 83]. Whether acting autocrinally or paracrinally, the neurotransmitters released by chromaffin cells (ATP, opioids and catecholamines) profoundly change Ca^{2+} channel gating, resulting in Ca^{2+} current depressions comparable in size to the blocking effects of selective Ca^{2+} channel antagonists [75, 77, 80]. This suggests that the endogenous modulation of Ca^{2+} channels can be functionally very effective in controlling exocytosis and that it may be relevant in identifying the role that each Ca^{2+} channel type plays in this process. This issue has become even more intriguing following the recently discovered differences between the endogenous inhibition of L- and non-L-channels induced by released neurotransmitters [3, 18, 20, 29, 57, 72].

Here, we review the basic aspects of L-channel modulation by focusing on the molecular and functional distinctions that characterize the main forms of this modulation. We discuss new convincing evidence in favour of voltage-independent L-channel inhibition mediated by $G_{i/o}$ proteins in chromaffin cells, which is autocrine, membrane-delimited and distinct from the voltage-dependent/cAMP-mediated facilitation of L-channels reported to occur in chromaffin cells of young cows [9, 10]. Together with the various forms of voltage-depen-

dent facilitation [35] the newly described voltage-independent inhibition seems designed to broaden the dynamic range of L-channel modulation in neurosecretory cells. Its autocrine nature and fast onset and offset appear suitable for the local control of Ca^{2+} entry under conditions in which secretion occurs at the resting potential or during mild depolarizations, at which voltage-gated L-channels predominate.

Multiple forms of L-channel gating modulation

L-channel modulation is largely heterogeneous and covers a broad spectrum of molecular mechanisms (see [36]). To limit our description to the more relevant ones, we review four types of modulatory pathways related to neuroendocrine L-channels: cAMP-mediated potentiation, voltage-dependent facilitation, voltage-dependent/cAMP-mediated phosphorylation and $G_{i/o}$ -protein-mediated inhibition. The first three types markedly enhance the L-channel current and are associated primarily with the cardiac and smooth muscle α_{1C} subunits and with the α_{1S} subunit of skeletal muscles (see [35] for a review). In contrast, the latter markedly depresses L-channel gating and seems mainly confined to the α_1 subunits of neuronal and neuroendocrine cells (α_{1D} , α_{1C}). There are not many reports on this latter type of modulation, which has previously been overlooked or considered of marginal interest. Recent findings at the single-channel level provide clear evidence of its existence in neuroendocrine cells [20] and of an important role in the control of Ca^{2+} signals in a number of cells.

The cAMP-mediated potentiation

Cardiac L-channel gating can be effectively potentiated by β -adrenergic stimulation, direct adenylate cyclase activation or application of membrane diffusible forms of cAMP [13, 90]. All three pathways increase intracellular cAMP, activate protein kinase A (PKA) and phosphorylate Ca^{2+} channels, even if the substrates responsible for the potentiating action are not yet fully defined [6, 62]. Production of cAMP through the activation of β_1 -adrenoceptors (β_1 -AR) requires 2–3 min to be completed, is mediated by G_s proteins coupled to adenylate cyclases and is mainly observed in cardiac cells that express high densities of L-channels and β_1 -AR. An increase in intracellular cAMP concentration markedly increases channel open probability (P_o) and the recruitment of newly functioning L-channels, with a resulting three- to four-fold increase of Ca^{2+} current amplitude [13]. The increased P_o is mainly due to a shift of channel gating from a low- P_o gating mode, displaying brief openings and long closures (mode 1), to a high- P_o gating mode with long lasting openings and short closures (mode 2). In many respects the action of cAMP resembles the potentiating action of Ca^{2+} channel agonists

[58], although the two mechanisms appear additive in enhancing the channel's mean P_o [97].

Voltage-dependent facilitation

Strong and long lasting depolarizations preceding a test pulse can induce large increases of L-currents in cardiac cells [85]. This facilitation is visible as long lasting openings on return to test potentials or as "slow tails" of increasing amplitude while prolonging the pre-pulse duration. The decay of the tail is strongly voltage-dependent and is accelerated at more negative test potentials. At -60 mV the "facilitation tail" is completed after about 20 ms and is absent or strongly reduced when using double-pulse protocols with inter-pulse repolarization to -60 mV for longer than 20 ms. The voltage-dependent facilitation is not limited to native cardiac L-channels. Neuronal and neuroendocrine L-channels [63, 68, 70] can also be facilitated according to the following rules: (1) the activation curve, $P_o(V)$, is shifted towards more negative potentials favouring large increases of P_o at potentials below $+10$ mV, (2) the degree of facilitation depends on the size and duration of the step depolarization ($\tau_{on} \cong 250$ ms at $+90$ mV), (3) the facilitation persists in the presence of cAMP or the kinase inhibitor H7 and does not require the phosphorylation of specific sites of the channel to develop [60, 68, 71], (4) the decay of facilitation is fast on return to very negative potentials and does not outlast channel closing [68]. It is fully prevented after 20-ms repolarizations below -60 mV [70].

The common explanation of this phenomenon is that long lasting depolarizations to positive voltages favour the switch of the channel from a low- to a high- P_o gating mode without apparently requiring the direct involvement of second messenger molecules [16, 23, 31, 38, 69, 71, 74]. This modulation does not produce evident kinetic changes to channel activation and is thus significantly different from the voltage-dependent facilitation of N- and P/Q-channels, which mainly delays channel openings [14, 17, 40]. Facilitation of N- and P/Q-channels involves the unbinding of a $G_o\beta\gamma$ subunit from the channel [54, 65] and does not require diffusible second messengers [59]. This modulation develops slowly at low voltages ($\tau_{on} \cong 40$ ms at -10 mV) but is very fast at strong positive potentials ($\tau_{on} \cong 5$ ms at $+90$ mV) and outlast channel closing [14, 40].

Although it is shown to occur in many preparations, the molecular basis of the voltage-dependent facilitation remains largely unclear. For instance, it is unknown whether it is due to an intrinsic property of the α_{1C} subunit or to the interaction of α_{1C} with the accessory subunits (β and $\alpha_2\text{-}\delta$). Recent studies on channel subunits expressed in heterologous systems only partially help to clarify this issue. Neuronal and cardiac α_{1C} isoforms require the co-expression of some β subunits to undergo voltage-dependent facilitation [16, 69, 89, 102] while the smooth muscle α_{1C} subunit alone accounts for the facilitation [74]. Of the four classes of identified β subunits,

only the neuronal β_{2a} prevents the voltage-dependent facilitation of cardiac α_{1C} [22]. In contrast, the remaining β subunits ($\beta_1, \beta_3, \beta_4$) and the cardiac β_{2a} are all permissive. In some cases the degree of facilitation is not long lasting on return to resting potentials (<50 ms) [69], while in others it is significantly longer than that observed in native L-channels (>200 ms) [86, 89]. Of some peculiarity is the rat brain β_{1b} whose co-expression with the rabbit cardiac α_{1C} produces a facilitation that lasts for tens of seconds on return to -90 mV [26].

The basis for these different actions seems related to the ability of the β subunit to affect the coupling between gating charge movement and channel opening [81]. For instance, neuronal β_{2a} increases the probability of cardiac α_{1C} to stay open in a conformational state that is similar to the facilitated state [27]. In this case, a voltage pre-pulse can not add any further degree of facilitation to channel gating and thus the current signal appears insensitive to voltage. The same occurs when the auxiliary $\alpha_2\text{-}\delta$ subunit is co-expressed with the cardiac α_{1C} and permissive β subunits [31]. The $\alpha_2\text{-}\delta$ subunit prevents the facilitation by shifting the channel to a conformational state in which the open facilitated state can be easily reached without a pre-pulse [86].

Voltage-dependent and cAMP-mediated phosphorylation

The voltage/cAMP-mediated phosphorylation is a mechanism by which L-channels are facilitated following a fast phosphorylation reaction favoured by strong depolarizations and by the close proximity of PKA to the channel. Obviously, this mechanism is significantly different from the voltage-dependent facilitation described above, but the end product is quite similar: a large current increment after a strong facilitatory pre-pulse. So far, voltage/cAMP-dependent phosphorylation has been reported for the native L-channels of bovine chromaffin cells [11] and skeletal muscles [67, 93] and for the cardiac and neuronal α_{1C} subunits expressed in heterologous systems [93, 95]. There are, however, several reports arguing against its existence in expression systems [16, 23, 31, 38, 74] and native L-channels [42, 60, 68, 71, 85], and there are even notable differences among the various types of voltage/cAMP-dependent phosphorylations that are not usually underlined but worth discussing.

The main characteristic of this modulation is that it disappears if either specific (PKI) or unspecific (H7, K252a) PKA inhibitors are applied to the cell. In skeletal muscles, PKI is sufficient to remove the pre-pulse-induced facilitation [94] while in chromaffin cells of young cows PKI is ineffective, suggesting the involvement of a pool of protein kinases responsible for the phenomenon [11]. The second main evidence is that compounds that increase the degree of channel phosphorylation, such as ATP[γ -S] and the phosphatase 2A inhibitor okadaic acid, produce steady increments of current amplitude with the consequent disappearance of the facilitation,

as if L-channels become persistently phosphorylated and thus facilitated. This point, however, is controversial. Cardiac, neuronal and smooth muscle α_{1C} subunits are sensitive to PKA but are not persistently facilitated by okadaic acid and ATP[γ -S], which preserve the voltage-dependent facilitation. This suggests that cAMP-dependent PKA activity mainly promotes L-channel up-regulation but not its voltage-dependent facilitation [16, 31, 38, 74].

A third property of this modulation is that neither GTP[γ -S] nor GDP[β -S] is able to potentiate or inhibit the facilitation of α_{1S} and α_{1C} subunits [93]. This, however, is not the case for chromaffin cells of young cows in which GTP[γ -S] and GDP[β -S] or the effects of receptor-activated $G_{i/o}$ proteins have not been tested directly. The only experiment disproving a role for G proteins is based on the removal of GTP from the recording pipette [11]. However, as noticed by Dolphin [35], removal of endogenous GTP may not be sufficient to uncouple highly expressed $G_{i/o}$ proteins from N- and P/Q-channels. This is a critical issue, since in the chromaffin cells of adult cows and other species, GTP[γ -S] and activated $G_{i/o}$ proteins produce marked slowing and depressions of N- and P/Q-currents, which can be quickly facilitated by conditioning pre-pulses [1, 2, 29, 37, 39, 45, 57, 72].

Finally, a significant peculiarity of voltage-dependent phosphorylation is that in some cases in order to develop it requires the anchoring of PKA near the channel by an A kinase anchoring protein (AKAP) [67]. The anchoring of PKA near to the channel would in fact be necessary for the fast voltage-dependent phosphorylation to occur in 50 ms [93]. In rabbit skeletal muscles there is evidence for the existence of a 15 kDa PKA-anchoring protein (AKAP15) that co-purifies and co-immunoprecipitates with the L-channel complex and plays a critical role in the voltage-dependent facilitation and regulation of skeletal muscle contraction [50, 51]. Whether this is valid for other cell preparations remains to be proven. Cardiac L-channels are also regulated by anchored PKA, but there is no indication that anchoring favours the degree of voltage/cAMP-dependent facilitation of these channels [47]. A small 80-residue AKAP (AKAP18) has been recently cloned from human brain and found to potentiate the activity of cardiac α_{1C} co-expressed with β_{2a} following exposure to cAMP analogues [44]. But, again there is no indication that AKAP18 acts on the voltage-dependent facilitation of cardiac L-channels.

In conclusion, there seems to be enough evidence supporting the existence of voltage-dependent phosphorylation of L-channels in skeletal muscles but a generalization to other L-channels, whether endogenous or transfected, seems unjustified. This is particularly true for bovine chromaffin cells in which the voltage-dependent phosphorylation of L-channels and the voltage-dependent facilitation of N- and P/Q-channels, producing nearly the same degree of Ca^{2+} current facilitation, raise some doubts about the co-existence of the two modulations in the same cell.

The $G_{i/o}$ -protein-mediated inhibition of neuroendocrine L-channels

Neuronal and neuroendocrine L-channels can be effectively inhibited by applied neurotransmitters through G-protein-mediated pathways. The action, however, appears quite variable. In most cases the inhibition is voltage-independent. There is no delay of L-channel activation and the depression mostly causes the scaling down of current [2, 8, 25, 43, 53, 78, 87, 91]. In other cases the neurotransmitters have no action on neuronal [28, 79] and neuroendocrine L-channels [96] and in some cases the inhibition is even voltage-dependent, closely resembling that of N- and P/Q-channels (see [35]). Despite this

variability the voltage-independent and G-protein-mediated inhibition of L-channels appears sufficiently widespread in neurons and neuroendocrine cells to be considered an interesting mechanism of neuronal Ca^{2+} signal modulation. This is particularly true for those cells in which L-channels are either highly expressed or are shown to control neurosecretion [48].

In bovine and rat chromaffin cells the G-protein-mediated inhibition of L-channels is mainly voltage-independent. The inhibition is triggered by the same neurotransmitters that are released by the secretory granules (ATP, opioids and catecholamines) and produces a scaling down of the current amplitude [2, 18, 57]. Figure 1 shows an example of the action of ATP and μ/δ opioid agonists (DAMGO and DPDPE) when applied directly to a rat chromaffin cell pre-treated with ω -conotoxin-GVIA (ω -CTx-GVIA) and ω -agatoxin-IVA (ω -Aga-IVA) to minimize N- and P/Q-currents and including Bay K 8644 to amplify the remaining L-current. Under these conditions the control currents (trace 1) are sensitive to nifedipine (trace 3), and strongly inhibited by mixtures of ATP and opioid agonists (DPDPE + DAMGO, trace 2). The inhibition causes a size reduction with no change in the activation time course, and a facilitatory pre-pulse to +100 mV is unable to recover the inhibition. The same occurs with mixtures of noradrenaline and adrenaline, suggesting that, as for the N- and P/Q-channels [3, 18, 29, 37], L-channel can also be autocrinally modulated by the neurotransmitters that are released by chromaffin cells. Indeed, in bovine [18] and rat chromaffin cells [57] the L-current changes its size depending on the rate of cell superfusion (Fig. 2). During "stop-flow" conditions the current is about half of the size recorded during rapid flow and a facilitatory pre-pulse to +100 mV is largely ineffective in recovering part of the depression.

As for the exogenous modulation, intracellular perfusions of GDP[β -S], cell pre-treatment with pertussis tox-

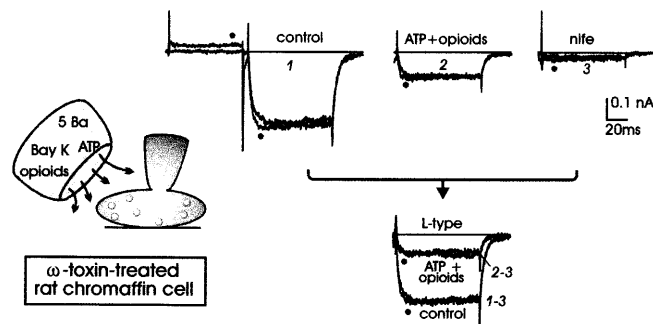
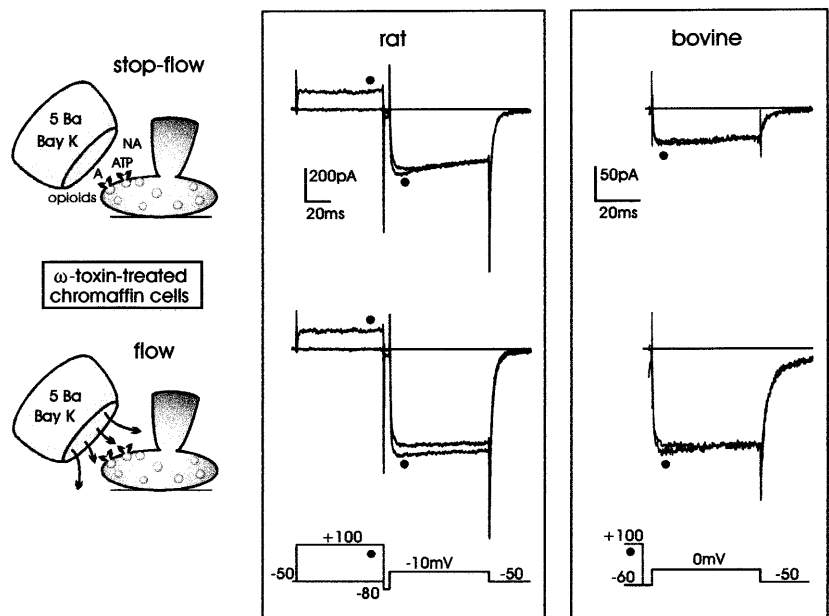


Fig. 1 L-currents inhibition by ATP and μ/δ -opioid agonists is insensitive to voltage in rat chromaffin cells. The cell was pre-treated with ω -CTx-GVIA, ω -Aga-IVA and Bay K 8644 and stimulated by a double-pulse protocol to test the existence of voltage-dependent modulation. The control currents (traces 1) are nearly halved by the mixture of agonists (traces 2) and almost fully blocked by addition of 5 μ M nifedipine (trace 3). This implies that most of the recorded currents are L-types as shown at the bottom after subtraction of nifedipine-resistant currents (traces 1-3 and 2-3). Notice that current activation is nearly identical before and after the facilitatory pre-pulse, indicating no signs of voltage-dependent facilitation of L-currents (modified from [57])

Fig. 2 L-currents of rat and bovine chromaffin cells are autocrinally inhibited by released neurotransmitters independently of voltage. In stop-flow condition (top) the currents are smaller than those recorded during fast superfusion (bottom). Notice the presence of a little voltage-dependent facilitation induced by the pre-pulse, particularly in rat chromaffin cells. The effect persisted in the presence of nifedipine (see Fig. 7 in [57]) and, thus, it is probably associated with R- rather than L-type channels (modified from [57] and [18])



in (PTX) and the application of autoreceptor antagonists prevent the autocrine inhibition of L-channels in rat chromaffin cells. Full removal of the stop-flow-induced inhibition requires the inclusion of purinergic, opioidergic and mixtures of adrenergic antagonists, suggesting that the autocrine modulation of L-channels is controlled by a set of autoreceptors ($P_{2x,y}$ purinergic, μ/δ opioidergic and α/β adrenergic) which, once activated, may sustain the endogenous inhibition of the current. The presence of a β -adrenoceptor coupled to $G_{i/o}$ proteins in chromaffin cells is not surprising since it is well known that cardiac myocytes and HEK293 cells express a β_2 -adrenoceptor (β_2 -AR) functionally coupled to both G_s and $G_{i/o}$ proteins [30, 100]. Thus, it is possible that rat chromaffin cells also possess a β_2 -AR in parallel with the α_2 -AR pharmacologically identified in the cow [73].

The existence of autocrine PTX-sensitive modulation of L-channels in bovine chromaffin cells is not novel. Indeed, it is indirectly suggested by the work of A. Feltz and collaborators, in which a remarkably fast up-regulation of L-currents in cells dialysed with intracellular solutions without GTP is shown [39]. In these experiments the cells were not superfused and the absence of GTP from the cytoplasm caused a twofold increase of L-currents. This can be explained by assuming that, during the first few minutes of recordings, L-channels are tonically inhibited by a pool of $G_{i/o}$ proteins activated by the released material and by the levels of endogenous GTP, which should be sufficiently high to keep the G proteins active. Wash out of GTP during cell dialysis removes the inhibition and causes the recovery of inhibited channels. Consistent with this, the up-modulation is faster when internal solutions contains GDP[β -S] and maximal in cells pre-treated with PTX.

Effective L-channel inhibition due to the feedback action of G proteins activated by the released material is also clear when L-currents are recorded from cells forming clusters [57]. Under these conditions, a single cell cannot be easily superfused and L-currents are abnormally low at the beginning of the recording, probably because of the inhibitory effects induced by the accumulation of released material. However, with GDP[β -S] inside the pipette the current progressively increases and reaches maximal values within 1 min.

The $G_{i/o}$ -dependent inhibition of neuroendocrine L-channels is "membrane delimited"

An important issue concerning the voltage-independent inhibition of L-channels in chromaffin cells is whether the mechanism requires some diffusible second messengers or is direct to the target channel (membrane delimited). A direct action of $G_{i/o}$ proteins on L-channels is perhaps indicated by the fast onset and offset of the inhibition during the rapid application and withdrawal of neurotransmitters to rat chromaffin cells [57]. The onset of the ATP-induced inhibition is rapid (τ_{on} 0.75 s) and the offset is complete within 20 s, i.e. comparable to that of

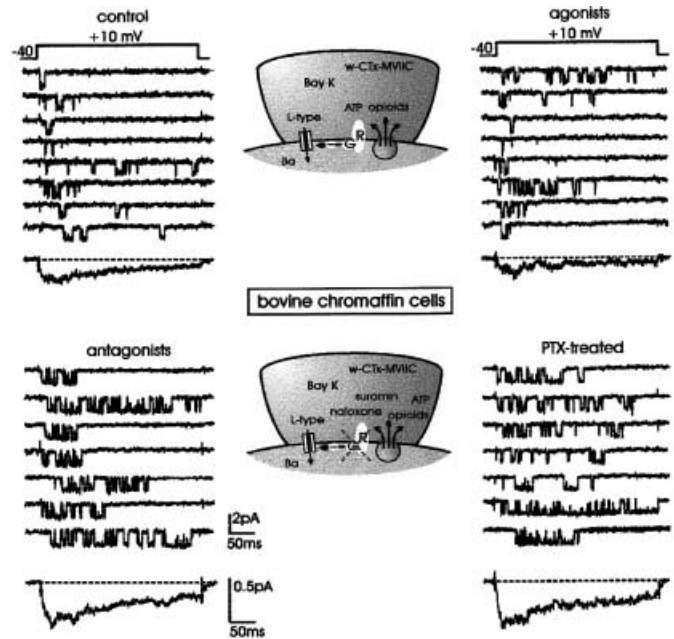


Fig. 3 Single L-channel inhibition in control conditions is mimicked by ATP and opioids and is prevented by receptor antagonists and pertussis toxin (PTX). Recordings were taken at +10 mV (V_h -40 mV) in different experimental conditions: at control (*top left*), with the agonists (100 μ M ATP, 10 μ M DAMGO and 1 μ M DPDPE) in the pipette (*top right*), in the presence of 100 μ M suramin and 10 μ M naloxone (*bottom left*) and after cell pre-treatment with PTX (*bottom-right*). The control pipette contained 100 mM BaCl₂, 5 μ M Bay K 8644 and 10 μ M ω -CTx-MV1IC to reduce the activity of non-L channels. In control patches the reduced channel activity is presumably due to the presence of the agonists released from the secretory granules. The traces at the *bottom* are averaged currents obtained from 150, 188, 166, and 125 sweeps, respectively. Notice the increased probability of opening in the presence of the antagonists and PTX and the similar activation kinetics of averaged currents in the four recording conditions (modified from [20])

neurotransmitter-induced inhibition of N-channels [14], which has been proven to be membrane delimited (see [59]). The most convincing evidence for a direct action, however, comes from recent single-channel studies of L-channel inhibition, in which the $G_{i/o}$ -protein-dependent inhibition of L-channels is shown to be autocrine and fully defined in cell-attached patches [20].

The main characteristics of this modulation at the single-channel level is illustrated in Fig. 3. In control conditions (with ATP and opioids released locally inside the pipette) or with the agonists added to the pipette, openings are rare and separated by long closures during depolarizations of 300 ms to +10 mV and the number of null sweeps is relatively large (33%) (see [20]). The probability of opening is low (P_o 0.27) despite the presence of Bay K 8644 in the pipette. The activity of single L-channels, however, is significantly enhanced if opioidergic and purinergic antagonists such as naloxone and suramin are included in the pipette or if the cells are pre-treated with PTX to prevent the activation of inhibitory $G_{i/o}$ proteins. Channel activity is clearly augmented under these

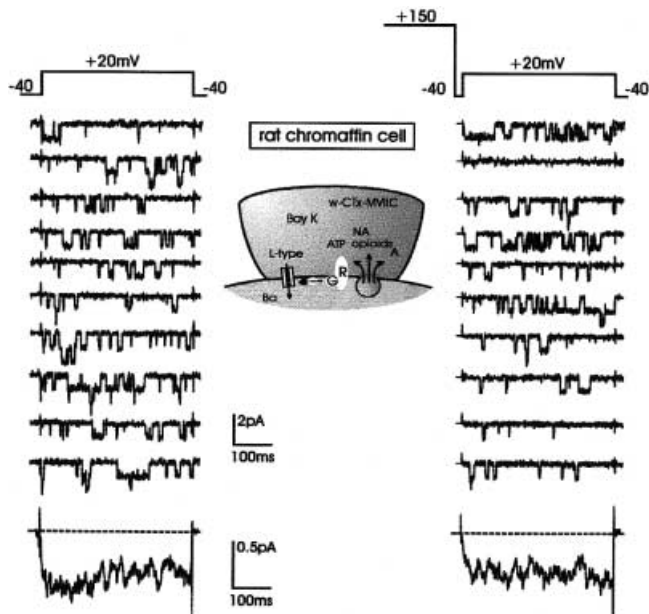


Fig. 4 Strong pre-pulses do not facilitate L-channel activity in cell-attached patches of rat chromaffin cells. Recordings to the *left* were alternated with those to the *right* and were separated by 6-s intervals. The pulse to +150 mV lasted 280 ms and the interpulse repolarization was 20 ms. The patch contained three channels as estimated by the simultaneous openings occurring during step depolarizations to +40 mV. The pipette contained 5 μ M Bay K 8644 and 10 μ M ω -CTx-MVHC. The *traces* at the *bottom* are averaged currents obtained from 30 sweeps. Notice that the averaged current after pre-pulse activates more slowly than that without a pre-pulse, as if the pre-depolarization inactivated a transient L-channel

conditions. With the antagonists, P_o increases from 0.27 to 0.41, the number of nulls decreases to 14% and averaged currents are nearly doubled. Analysis of open and closed time distributions shows that the enhanced P_o is mostly due to shorter mean closed times rather than larger mean open times, suggesting an average higher frequency of openings rather than an increased open channel duration.

The results of Fig. 3 allow some interesting conclusions to be drawn. First, as for the N- and P/Q-channels [18], the L-channel activity in control patches is strongly conditioned by the endogenous inhibition due to the material released inside the pipette during patch stimulation (local release). The addition of saturating doses of agonists (ATP and opioids) inside the pipette causes a comparable degree of inhibition, suggesting that secretion induced by Bay-K-8644-modified L-channels occurs in a high percentage of patches. Second, the inhibition of L-channels in bovine chromaffin cells is fully defined in membrane patches of $\approx 1 \mu\text{m}^2$ diameter and thus there is no requirement for a diffusible second messenger. This conclusion is also supported by the fact that applying saturating doses of ATP and opioids outside the patch is unable to depress L-channel activity within minutes of continuous superfusion. Third, the local inhi-

bition does not alter the channel activation kinetics, suggesting that neurotransmitters do not induce delayed openings. Thus, unlike the N- and P/Q-channels [17, 18], there is no voltage dependence associated with the changes of channel gating. This is better proved by the absence of any facilitation following double-pulse protocols. Figure 4 shows an example of recordings from a cell-attached patch with three L-channels recorded from a rat chromaffin cell under control conditions. Obviously, the pre-pulse does not induce any sign of voltage-dependent facilitation. On average, channel activity is preserved and there is no acceleration of channel activation after the pre-pulse. If anything, the averaged current after the pre-pulse is slightly delayed, as if the patch contained a fast inactivating channel no longer available after strong and prolonged pre-conditioning pulses. Similar findings have been reported for bovine chromaffin cells [20].

The molecular components of the G-protein-induced L-channel inhibition

A direct modulation of inhibitory G proteins on L-channels raises interesting questions about the possible interaction between $G_{i/o}$ proteins and the α_1 subunit of neuroendocrine L-channels. The first question concerns the possibility that the voltage-independent mechanism may closely resemble the voltage-dependent inhibition of N- and P/Q-channels induced by $G_o\beta\gamma$ subunits. The latter is clearly due to an interaction between $G_o\beta\gamma$ and the I-II intracellular loop of the α_{1A} (α_{1B}) subunit [34, 101]. The binding of $G_o\beta\gamma$ is apparently conditioned by the presence of the regulatory β channel subunits, which may compete for the same site on the α_{1A} (α_{1B}) pore subunit. Unlike α_{1C} channel facilitation, in which the β subunit favours the modulation, in the case of α_{1A} (α_{1B}), the channel β subunit prevents the binding of $G_o\beta\gamma$ and thus the development of inhibition. Notice that the binding of $G_o\beta\gamma$ is associated with the presence of a QQIER motif in the I-II loop of α_{1A} (α_{1B}) and a QQLEE motif is present in both the α_{1D} and the α_{1C} subunits [55]. Thus, it might be possible that the G-protein-dependent inhibition of L-channels is linked to the same I-II loop region of the α_1 pore. It would be interesting to see how the voltage-independent modulation of the channel is exerted and by which G protein subunit it is controlled. It could also be that, unlike the N- and P/Q-channels, modulation of the L-channel is not associated with a $G\beta\gamma$ subunit but rather with a $G\alpha$ subunit. Presently there are no clear indications about the molecular mechanism and the type of G protein involved. Notice, however, that the voltage-independent component of N-current inhibition in sympathetic neurons is associated with $G_i\beta\gamma$ subunits while the voltage-dependent component is mediated by $G_o\beta\gamma$ [33]. The same could be true for L-channel inhibition, but whether by $G_i\beta\gamma$, $G_o\beta\gamma$, $G_i\alpha$ or $G_o\alpha$ remains to be proved.

Voltage-independent inhibition versus cAMP-dependent facilitation in chromaffin cells

The presence of voltage-independent inhibition of single L-channels in control patches of bovine [18, 20] and rat chromaffin cells sharply contrasts with the existence of a long lasting voltage-dependent facilitation of L-channels, as in chromaffin cells of young cows [9, 10, 11, 12]. This facilitation has impressive singularities that differ markedly not only from the voltage-dependent facilitation of N-channels but also from the various L-channel facilitation of cardiac, neuronal and neuroendocrine cells [16, 64, 74, 85, 93]. In brief the characteristics of the “facilitation L-current” are as follows: (1) it develops quickly at very positive potentials (≈ 50 ms at +100 mV) and helps to recover the fast kinetics of the slowly activating Ca^{2+} currents, (2) the facilitation lasts for a few seconds on return to very negative potentials, (3) it is prevented by Bay K 8644, cAMP, phosphatases inhibitors and ATP[γ -S], (4) it is, however, not prevented by a specific inhibitor of PKA, suggesting the involvement of a pool of protein kinases.

In the chromaffin cells of adult cows, Ca^{2+} channel modulation appears significantly different from that reported by Artalejo et al. [9, 10] at both the macroscopic and single-channel level. Most of the voltage-dependent facilitation persists in the presence of DHP-antagonists and is due to the removal of an autocrine inhibition of N- and P/Q-channels [1, 2, 3, 18, 29, 37, 45, 72, 83]. This facilitation develops quickly, recovers the fast activation of Ca^{2+} current and produces a 30–40% increase of its peak amplitude. So, at least in the chromaffin cells of adult cows and other animal species [61, 83] there seems to be little space for L-channel facilitation as described in young cows. Even at the single-channel level there is no evidence for this current before and after pre-pulses [15, 18].

Hoshi and Smith [64] and Hoshi et al. [63] reported on the voltage-dependent facilitation of L-channels in bovine chromaffin cells but the characteristics of this modulation were significantly different from those measured in young cows. In one case [64], the facilitation was observed at the single-channel level in the presence of Bay K 8644, as “tail openings” on return to test potentials of 0 to +25 mV after pre-pulses to +80 mV. The “tail openings” were clearly from Bay-K-8644-modified L-channels, but they were significantly short on return to negative voltages, suggesting that facilitation was short lasting and similar to the voltage-dependent facilitation described by Pietrobon and Hess [68, 70, 85]. In the second case [63], the facilitation was resolved in whole-cell recordings in the absence of specific Ca^{2+} channel blockers. Under these conditions, Ba^{2+} currents in bovine chromaffin cells are predominantly through N- and P/Q-channels (80%) and, thus, it is unlikely that the observed 30–40% facilitation of the current could be associated with L-channels, which contribute only 10–20% of the total current [2, 3]. Very likely, the facilitation observed by Hoshi et al. [63] was due to the voltage-dependent re-

covery of non-L-channels that had been inhibited by locally released neurotransmitters. Notice that control currents during test pulses were slowly activating and accelerated after the pre-pulse (Fig. 1 in [63]), as expected by the endogenous modulation of N- and P/Q-channels [3, 29, 37, 45].

In our experience [18] the voltage-dependent facilitation of single L-channels of bovine chromaffin cells is not easily detectable in control conditions (i.e. in the absence of Bay K 8644), even without brief repolarizations between test pulses and pre-pulses. We found evidence for the voltage-dependent facilitation only in the presence of Bay K 8644 and ω -CTx-MVIIC, which enhance the contribution of L-channels. The facilitation was evident with inter-pulses shorter than 10–20 ms and test depolarizations below 0 mV, but was prevented by inter-pulses longer than 20 ms at –60 mV [20]. This may suggest the presence of a short lasting voltage-dependent facilitation that does not outlast channel closing [68, 70, 85], but we attributed part of the facilitation to a direct action of the DHP on L-channel gating. Indeed, in RINm5F cells containing large percentages of L-channels, Bay K 8644 and other DHP agonists are able to induce a variable delay of L-channel activation and exhibit voltage-dependent facilitation with double-pulse stimulation [49, 98].

Coexistence of the $G_{i/o}$ -protein-induced inhibition and cAMP-mediated potentiation of L-channel gating in chromaffin cells

The existence of direct $G_{i/o}$ -mediated modulation and the absence of long lasting PKA-dependent facilitation of L-channels in bovine [18, 19, 20] and rat chromaffin cells [57] does not exclude the possibility that these channels are also modulated by a diffusible cAMP/PKA-dependent mechanism [70, 90] capable of interfering with other existing modulatory pathways. Indeed, this is what happens in bovine and rat chromaffin cells when L-channel modulation is studied in cell-attached patches to prevent the loss of intracellular regulatory subunits, which occurs in whole-cell recordings [21].

As shown in Fig. 5, applying membrane-permeable forms of cAMP (8-CPT-cAMP) can effectively potentiate the activity of Bay-K-8644-modified L-channels in the presence of exogenous or released agonists [20]. Cell incubation with 1 mM 8-CPT-cAMP produces an increased number of traces with high P_o (from 0.2 to 0.42) and reduces the number of nulls (from 41% to 13%), which justifies the approximately threefold increase of mean current amplitude with cAMP.

A detailed analysis of cAMP action shows that the increased P_o is mainly due to prolonged mean open times (t_o from 3.4 ms to 5.4 ms at +10 mV) and shorter mean closed times (t_c from 22.8 ms to 10.1 ms). The single L-channel conductance is not altered by cAMP while proportional changes of P_o , t_o and t_c are observed at every potential. P_o reaches maximal values of 0.73 at

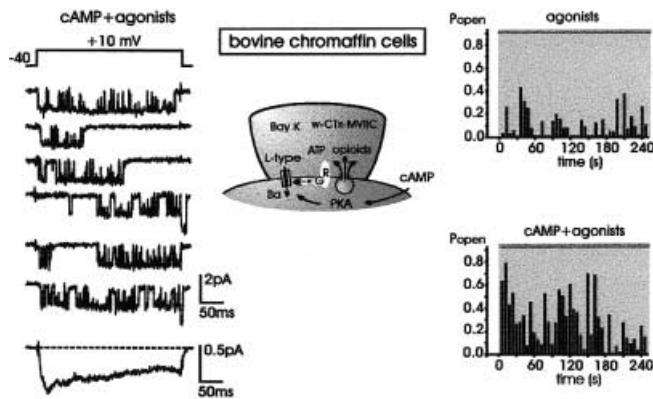


Fig. 5 The membrane-permeable cAMP analogue (8-CPT-cAMP) increases the activity of L-channels. To the left are representative recordings taken from a patch exposed to 1 mM 8-CPT-cAMP. The bottom trace is the averaged current obtained from 164 sweeps. To the right are shown the open probability (P_o) calculated from consecutive sweeps in the presence (bottom) or absence (top) of 8-CPT-cAMP (50 μ M) and with the agonists in the pipette (modified from [20])

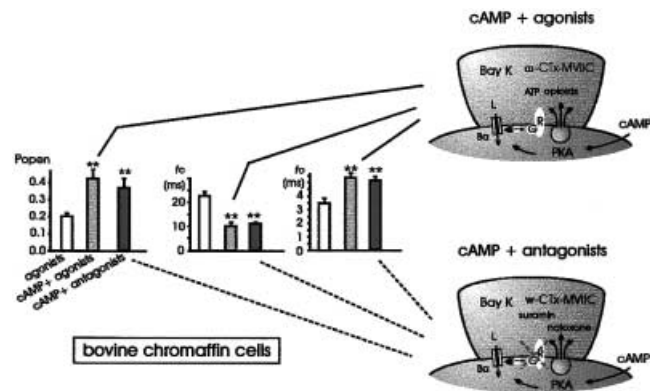


Fig. 6 cAMP-mediated potentiation persists regardless of the presence of $G_{i/o}$ protein activation. To the left are shown the mean P_o , mean closed (t_c) and mean open (t_o) times at +10 mV obtained from patches of bovine chromaffin cells containing single Bay-K-8644-modified L channels. The pipette contained either a mixture of agonists (ATP + opiates) or antagonists (suramin + naloxone). 8-CPT-cAMP (1 mM) was added to the bath. (Modified from [20])

+30 mV, which is significantly larger than the P_o attained in the presence of the agonists at the same potential (mean P_o 0.4). Most interestingly, the cAMP-induced potentiation of L-channels occurs in the presence of the agonists and remains similarly elevated by replacing the agonists with antagonists (Fig. 6) or in patches pre-treated with PTX [20]. This suggests that cAMP potentiates L-channel gating activity regardless of the inhibitory action of $G_{i/o}$ proteins and that the two modulations (inhibition and potentiation) may converge on a single target. As for other L-channels [60, 70], cAMP had no effects on the voltage dependence of L-channel modulation. Double-pulse protocols of the type used in Fig. 4 give very similar effects with cAMP, i.e. no voltage-dependent facilitation of available channels [20].

Most likely, the action of cAMP develops through a PKA-mediated pathway. In fact mixtures of forskolin and isobutylmethylxanthine (IBMX) in bovine chromaffin cells mimic the effects of cAMP and applications of the protein kinase inhibitor H7 and H89 prevent the action of forskolin + IBMX or cAMP alone. Under these conditions, the most obvious conclusion is that a cAMP/PKA pathway exists in adult bovine chromaffin cells which, regardless of the presence of active $G_{i/o}$ proteins, is able to up-regulate the gating of the available L-channels.

Given that the autocrine inhibition is effective under control conditions and absent during maximal activation of the cAMP/PKA pathway, the next obvious question is whether the $G_{i/o}$ -protein-induced inhibition remains functional when the cAMP-mediated potentiation is turned off. This can be tested by studying the effects of agonists, antagonists and PTX on L-channels in cells pre-treated with H7 in which the PKA-mediated potentiation is abolished. Indeed, in the three conditions the P_o at +10 mV has more or less the same values of patches without H7, i.e. $P_o \approx 0.2$ under control conditions and about a factor 2 larger with the antagonists or in the presence of PTX (Fig. 7). This suggests two important conclusions. The first is that the actions of cAMP and $G_{i/o}$ proteins are distinct and not additive: one of the two pathways can always function while the other one is inactive and, in all cases, cAMP up-regulates the gating of L-channels regardless of the presence of activated $G_{i/o}$ proteins. The second is that bovine chromaffin cells possess low basal levels of active PKA and cAMP, which keep the available L-channels down-regulated. This, in addition to the $G_{i/o}$ -protein-induced inhibition triggered by the endogenous release of neurotransmitters, switches the channel into a gating mode of very low P_o at rest.

A model for the G-protein- and cAMP-dependent modulation of L-channels

The above results suggest a simplified scheme for the L-channel modulation in chromaffin cells (Fig. 8). These cells express high densities of purinergic and opioidergic autoreceptors (R) as well as a sufficient density of L-channels [1, 46, 61]. These conditions favour the close coupling between Ca^{2+} channels and $G_{i/o}$ proteins ($G_{i/o}$) coupled to opioidergic and purinergic autoreceptors activated by locally released neurotransmitters. With a frequency of four to eight secretory events per minute [4, 92] and with the secretory granules locally releasing high concentrations of ATP and opiates [99], it is likely that in a significant percentage of patches (30–40%) the pipette accumulates a sufficient amount of neurotransmitters to activate the autoreceptors and keep the L-channel in a low- P_o gating mode. In parallel to this, local or remote receptor-mediated pathways can drive the activity of adenylate cyclase (AC) and potentiate, via the cAMP/PKA pathway, the channel gating by phos-

Fig. 7 The agonist-induced L-channel inhibition persists when the cAMP pathway is abolished. Single L-channel activities at +10 mV were recorded from patches pre-treated with 200 μ M H7 with the agonists (ATP + opioids) (*top*) or the antagonists (suramin + naloxone) in the pipette (*bottom*). The averaged currents on the *right* were calculated over 180 and 153 sweeps pooled from 7 and 4 patches, respectively. On the *left*, mean P_o at +10 mV calculated from $n=11$ patches (agonists + H7), $n=4$ (antagonists + H7) (** $P<0.01$) and $n=5$ (PTX + H7) (** $P<0.01$). (Modified from [20])

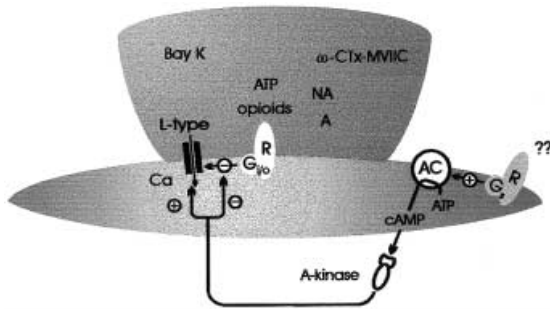
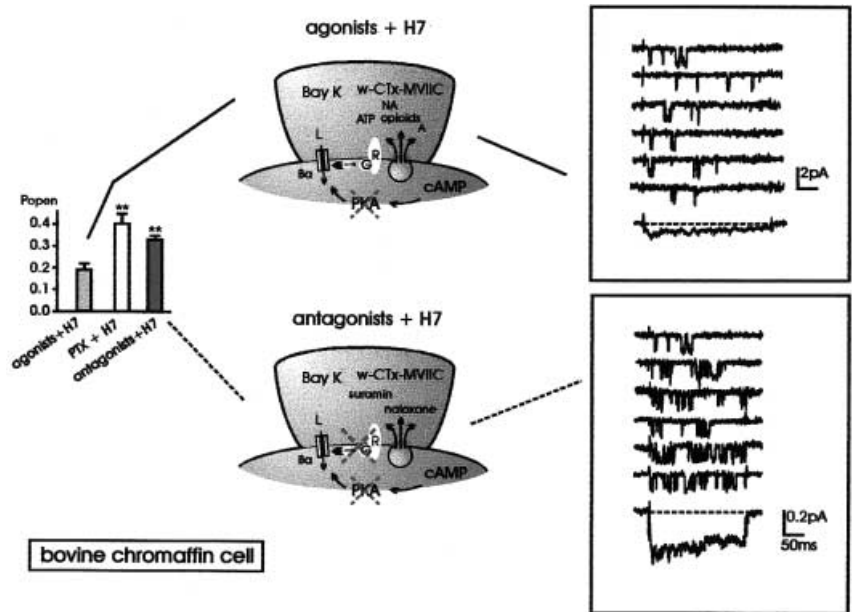


Fig. 8 A model for local $G_{i/o}$ -protein-induced inhibition and cAMP-mediated potentiation of L-channels in chromaffin cells. The L-channel can be inhibited by a PTX-sensitive G protein, or by the activation of μ/δ -opioidergic and $P_{2x,y}$ -purinergic receptors (R). The channel can also be potentiated by the phosphorylating action of PKA on the channel subunit. The phosphorylation by cAMP capable of preventing the action of the inhibitory G protein cannot be excluded [66]. The cAMP-mediated phosphorylation of the channel occludes the inhibitory effects induced by the agonists [ATP, opioids, noradrenaline (NA), adrenaline (A)] and switches the channel in to a high- P_o gating mode. (AC adenylyl cyclase)

phorylating the channel subunits. Obviously, a concomitant negative effect of PKA phosphorylation on $G_{i/o}$ proteins cannot be excluded [66].

An open question is which receptors are most likely associated with the G_s protein positively coupled to AC and under what circumstances is the cAMP-mediated pathway active. Bovine chromaffin cells express a variety of receptors, which may be associated with AC and thus with changes of cAMP (PACAP, D1, GABA_B, VIP, β -AR) but little is known about their action on Ca^{2+} channels, except for the up-regulatory effects of D1 receptors reported by Artalejo et al. [9] in their study of young bovine chromaffin cells. The existence and the

physiological role of D1 receptors in chromaffin cells, however, is controversial [9, 61, 76] and the same uncertainty exists about the presence and functioning of β -ARs [73, 82, 84]. Possibly, most of these discrepancies derive from differences in experimental conditions, cell preparations and species-specificity. A crucial one concerns the Ca^{2+} current recordings in intact versus dialysed cells (cell-attached versus whole-cell recordings), which can significantly alter the intracellular content of the cell.

This could be particularly critical when dealing with modulations involving catalytic subunits and diffusible second messengers. For instance, the recently described negative action of β -AR on whole-cell L currents of rat chromaffin cells [57] may give a distorted view of the role of these receptors if the action of β -ARs is not tested in intact cells at the single-channel level as well. In fact, in a preliminary series of experiments on rat chromaffin cells (T. Cesetti & E. Carbone, unpublished observations) we found that in four out of ten patches the application of isoprenaline (10 μ M) outside the patch induces the up-regulation of single L-channels and propranolol (1 μ M) reverts the action, although with some delay (Fig. 9). Thus, chromaffin cells may possess multiple β -ARs, which can up-regulate L-channels by a remote cAMP/PKA control (β_1 -AR) [24], or inhibit the channels through $G_{i/o}$ proteins when the G_s proteins are uncoupled by receptor desensitization (β_2 -AR) [30]. The inhibition may occur also if the cAMP pathway is down-regulated during cell dialysis. These findings also suggest that the up-regulation of L-channels might not only have a paracrine origin through the release of non-cholinergic neurotransmitters from sympathetic terminals [52, 88], but could also derive from a feedback mechanism mediated by β -AR whose complex action remains to be fully understood.

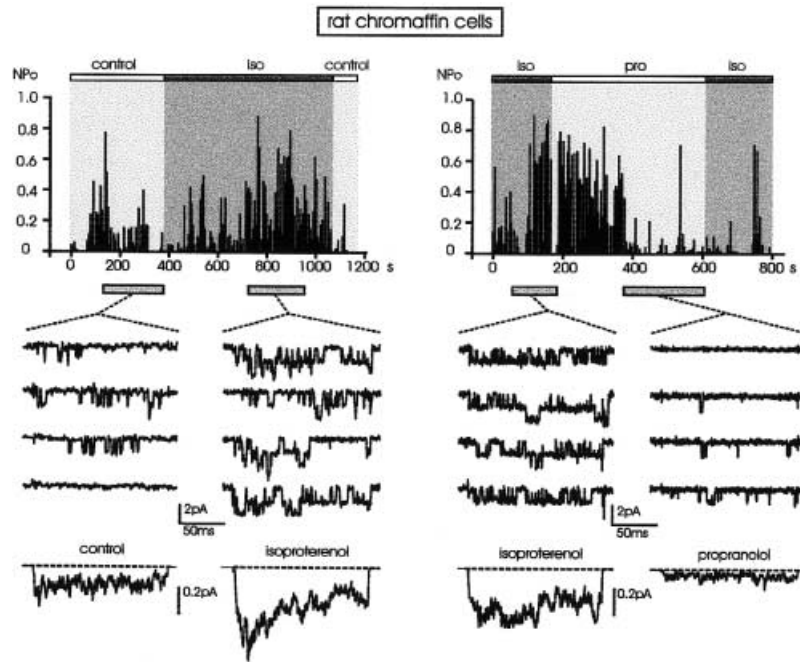


Fig. 9 Remote applications of isoprenaline up-regulate L-channel activity in rat chromaffin cells and propranolol prevents the action. On the *top left* are plotted the NP_o values at +10 mV monitored sweep by sweep from a cell sequentially perfused with a control solution, isoprenaline (10 μ M) and control again. The cell-attached patch contained three channels whose activity became more evident during isoprenaline application. Representative traces and averaged currents at the time intervals indicated are given below. The averaged currents were obtained by averaging $n=50$ (control) and $n=40$ idealized sweeps (*iso*). To the *right* are shown data from a cell-attached patch containing two channels that were first exposed to isoprenaline (10 μ M) and subsequently to propranolol (1 μ M) and then again to isoprenaline. Notice that L-channel activity is nearly abolished by the β -adrenoceptors (β -AR) antagonist. Averaged currents were obtained from $n=32$ (*iso*) and $n=60$ idealized sweeps (*pro*)

Conclusions

The existence of a direct autocrine inhibition of neuroendocrine L-channels by PTX-sensitive G proteins opens new and interesting considerations about the role of these channels in the control of neurosecretion. The first consequence of these two opposite mechanisms is that, besides being potentiated by a cAMP-dependent mechanism, neuroendocrine L-channels can be also down-modulated by a fast and reversible feedback mechanism, which can halve the percentage of basal L-channels available at rest. This implies that L-channels may be controlled by a wide range of mechanisms, which could be part autocrine and part paracrine. The two combined modulations ($G_{i/o}$ -protein-induced inhibition and cAMP-mediated potentiation) may be critical for the fine tuning of neurosecretion in intact adrenal glands [48]. These properties are probably applicable to other neuroendocrine and neuronal systems in which L-channels are proved crucial for triggering hormone release and nuclear events associated with gene expression [32, 41].

Acknowledgements This work was funded by the Italian MURST and supported by a NATO grant to E.C. (# CRG 972224). J.M. H-G. is supported by a Marie Curie EU postdoctoral fellowship.

References

- Albillos A, Artalejo AR, López MG, Gandía L, García AG, Carbone E (1994) Ca^{2+} channel subtypes in cat chromaffin cells. *J Physiol (Lond)* 477:197–213
- Albillos A, Carbone E, Gandía L, García AG, Pollo A (1996) Opioid inhibition of Ca^{2+} channel subtypes in bovine chromaffin cells: selectivity of action and voltage-dependence. *Eur J Neurosci* 8:1561–1570
- Albillos A, Gandía L, Michelena P, Gilabert J-A, del Valle M, Carbone E, García AG (1996) The mechanism of calcium channel facilitation in bovine chromaffin cells. *J Physiol (Lond)* 494:687–695
- Albillos A, Dernick G, Horstmann H, Almers W, Alvarez de Toledo G, Lindau M (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* 389:509–512
- Albillos A, Neher E, Moser T (2000) R-Type Ca^{2+} channels are coupled to the rapid component of secretion in mouse adrenal slice chromaffin cells. *J Neurosci* 20:8323–8330
- Allen TJA, Mikala G, Wu X, Dolphin AC (1998) Effects of 2,3-butanedione-monoxime (BDM) on calcium channels expressed in *Xenopus* oocytes. *J Physiol (Lond)* 508:1–14
- Almers W (1990) Exocytosis. *Annu Rev Physiol* 52:607–624
- Amico C, Marchetti C, Nobile M, Usai C (1995) Pharmacological types of calcium channels and their modulation by baclofen in cerebellar granules. *J Neurosci* 15:2839–2848
- Artalejo CR, Ariano MA, Perlman RA, Fox AP (1990) Activation of facilitation calcium channels in chromaffin cells by D_1 dopamine receptors through a cAMP/protein kinase A-dependent mechanism. *Nature* 348:239–242
- Artalejo CR, Mogul DJ, Perlman RA, Fox AP (1991) Three types of bovine chromaffin cell Ca^{2+} channels: facilitation increases the opening probability of a 27 pS channel. *J Physiol (Lond)* 444:213–240
- Artalejo CR, Rossie S, Perlman RA, Fox AP (1992) Voltage-dependent phosphorylation may recruit Ca^{2+} current facilitation in chromaffin cells. *Nature* 358:63–66

12. Artalejo CR, Adams ME, Fox AP (1994) Three types of Ca²⁺ channel trigger secretion with different efficacies in chromaffin cells. *Nature* 367:72–76
13. Bean BP, Nowycky MC, Tsien RW (1983) β -Adrenergic modulation of calcium channels in frog ventricular heart cells. *Nature* 307:371–375
14. Boland L, Bean BP (1993) Modulation of N-type calcium channels in bullfrog sympathetic neurons by luteinizing hormone-releasing hormone: kinetics and voltage-dependence. *J Neurosci* 13:516–533
15. Bossu J-L, De Waard M, Feltz A (1991) Two types of calcium channels are expressed in adult bovine chromaffin cells. *J Physiol (Lond)* 437:621–634
16. Bourinet E, Charnet P, Tomlinson WJ, Stea A, Snutch TP, Nargeot J (1994) Voltage-dependent facilitation of a neuronal a_{1C} L-type calcium channel. *EMBO J* 13:5032–5039
17. Carabelli V, Lovallo M, Magnelli V, Zucker H, Carbone E (1996) Voltage-dependent modulation of single N-type Ca²⁺ channel kinetics by receptor agonists in IMR32 cells. *Biophys J* 70:2144–2154
18. Carabelli V, Carra I, Carbone E (1998) Localized secretion of ATP and opioids revealed through single Ca²⁺ channel modulation in bovine chromaffin cells. *Neuron* 20:1255–1268
19. Carabelli V, Carbone E (2000) Direct autocrine inhibition and cAMP-dependent potentiation of single L-type Ca²⁺ channels in bovine chromaffin cells. *Biophys J* 78:A103
20. Carabelli V, Hernández-Guijo JM, Baldelli P, Carbone E (2001) Direct autocrine inhibition and cAMP-dependent potentiation of single L-type Ca²⁺ channels in bovine chromaffin cells. *J Physiol (Lond)* 532:73–90
21. Carbone E, García AG (1997) More on calcium channels. *Trends Neurosci* 20:448–450
22. Cens T, Mangoni ME, Richard S, Nargeot J, Charnet P (1996) Coexpression of the β_2 subunit does not induce voltage-dependent facilitation of the class C L-type Ca channel. *Pflügers Arch* 431:771–774
23. Cens T, Restituito S, Vallentin A, Charnet P (1998) Promotion and inhibition of L-type Ca²⁺ channel facilitation by distinct domains of the β subunit. *J Biol Chem* 273:18308–18315
24. Chen-Izu Y, Xiao R-P, Izu LT, Cheng H, Kuschel M, Spurgeon H, Lakatta EG (1995) Gi-dependent localization of β_2 -adrenergic receptor signaling to L-type Ca²⁺ channels. *Biophys J* 79:2547–2566
25. Ciranna L, Feltz P, Schlichter R (1996) Selective inhibition of high voltage-activated L-type and Q-type Ca²⁺ currents by serotonin in rat melanotrophs. *J Physiol (Lond)* 490:595–609
26. Costantin JL, Qin N, Zhou J, Platano D, Birnbaumer L, Stefani E (1998) Long lasting facilitation of the rabbit cardiac Ca²⁺ channel: correlation with the coupling efficiency between charge movement and pore opening. *FEBS Lett* 423:213–217
27. Costantin JL, Noceti F, Qin N, Wei X, Birnbaumer L, Stefani E (1998) Facilitation by the β_{2a} subunit of pore openings in cardiac Ca²⁺ channels. *J Physiol (Lond)* 507:93–103
28. Cox DH, Dunlap K (1992) Pharmacological discrimination of N-type from L-type Ca²⁺ current and its selective modulation by neurotransmitters. *J Neurosci* 12:906–914
29. Currie KPM, Fox AP (1996) ATP serves as a negative feedback inhibitor of voltage-gated Ca²⁺ channel currents in cultured bovine chromaffin cells. *Neuron* 16:1027–1036
30. Daaka Y, Luttrell LM, Leifkowitz RJ (1997) Switching of the coupling of the β_2 -adrenergic receptor to different G proteins by protein kinase A. *Nature* 390:88–91
31. Dai SD, Klugbauer N, Zong X, Seisenberger C, Hofmann F (1999) The role of subunit composition on prepulse facilitation of the cardiac L-type calcium channel. *FEBS Lett* 442:70–74
32. Deisseroth K, Heist EK, Tsien RW (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* 392:198–202
33. Delmas P, Abogadie FC, Milligan G, Buckley NJ, Brown DA (1999) β dimers derived from G_o and G_i proteins contribute different components of adrenergic inhibition of Ca²⁺-channels in rat sympathetic neurones. *J Physiol (Lond)* 518:23–26
34. De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP (1997) Direct binding of G protein by complex to voltage-dependent calcium channels. *Nature* 385:446–450
35. Dolphin A (1996) Facilitation of Ca²⁺ currents in excitable cells. *Trends Neurosci* 19:35–43
36. Dolphin A (1999) L-type calcium channel modulation. *Adv Sec Mess Phosphoprot Res* 33:153–170
37. Doupnik CA, Pun RYK (1994) G-protein activation mediates prepulse facilitation of Ca²⁺ channel currents in bovine chromaffin cells. *J Membr Biol* 140:47–56
38. Eisfeld J, Mikala G, Schwartz A, Varadi G, Klöckner U (1996) Lack of involvement of protein kinase A phosphorylation in voltage-dependent facilitation of the activity of human cardiac L-type calcium channels. *Biochem Biophys Res Commun* 221:446–453
39. Elhamdani A, Bossu J-L, Feltz A (1995) ATP and G-proteins affect the run up of the Ca²⁺ current in bovine chromaffin cells. *Pflügers Arch* 430:410–419
40. Elmslie KS, Zhou W, Jones SW (1990) LHRH and GTP- γ -S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* 5:75–80
41. Fass DM, Talimoto K, Mains RE, Levitan ES (1999) Tonic dopamine inhibition of L-type Ca²⁺ channel activity reduces a_{1D} Ca²⁺ channel gene expression. *J Neurosci* 19:3345–4452
42. Fleig A, Penner R (1995) Excessive repolarization-dependent calcium currents induced by strong depolarization in rat skeletal myoballs. *J Physiol (Lond)* 489:41–53
43. Formenti A, Martina M, Plebani A, Mancina M (1998) Multiple modulatory effects of dopamine on calcium channel kinetics in adult rat sensory neurons. *J Physiol (Lond)* 509:395–409
44. Fraser IDC, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marrion NV, Scott JD (1998) A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. *EMBO J* 17:2261–2272
45. Gandía L, García AG, Morad M (1993) ATP modulation of calcium channels in chromaffin cells *J Physiol (Lond)* 470:55–72
46. Gandía L, Borges R, Albillos A, García AG (1995) Multiple calcium channel subtypes in isolated rat chromaffin cells. *Pflügers Arch* 430:55–63
47. Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD, Hosey MM (1997) cAMP-dependent regulation of cardiac L-type Ca²⁺ channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* 19:185–196
48. García AG, Sala F, Reig JA, Viniegra S, Frías J, Fonteriz RI, Gandía L (1984) Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. *Nature* 309:69–71
49. Giusta L, Ferrero L, Visentin S, Gasco A, Carbone E (1999) Voltage-dependent inhibition of L-type Ca channels induced by new and classical DHP-agonists in RINM5F cells. *Biophys J* 76:A342
50. Gray PC, Tibbs VC, Catterall WA, Murphy BJ (1997) Identification of a 15-kDa cAMP-dependent protein kinase-anchoring protein associated with skeletal muscle L-type calcium channels. *J Biol Chem* 272:6297–6302
51. Gray PC, Johnson BD, Westenbroek RE, Hays LG, Yates III JR, Scheuer T, Catterall WA, Murphy BJ (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels. *Neuron* 20:1017–1026
52. Hahm SH, Hsu CM, Eiden LE (1998) PACAP activates calcium influx-dependent and -independent pathways to couple met-enkephalin secretion and biosynthesis in chromaffin cells. *J Mol Neurosci* 11:43–56
53. Haws CM, Slesinger PA, Lansman JB (1993) Dihydropyridine- and ω -conotoxin-sensitive Ca²⁺ currents in cerebellar neurons: persistent block of L-type channels by a pertussis toxin-sensitive G-protein. *J Neurosci* 13:1148–1156
54. Herlitze S, García DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca²⁺ channels by G-protein by subunits. *Nature* 380:58–62
55. Herlitze S, Hockerman GH, Scheuer T, Catterall WA (1997) Molecular determinants of inactivation and G protein modula-

- tion in the intracellular loop connecting domains I and II of the calcium channel α_{1A} subunit. *Proc Natl Acad Sci USA* 94:1512–1516
56. Hernández-Guijo JM, de Pascual R, García AG, Gandía L (1998) Separation of calcium channel current components in mouse chromaffin cells superfused with low- and high-barium solutions. *Pflügers Arch* 436:75–82
 57. Hernández-Guijo JM, Carabelli V, Gandía L, García AG, Carbone E (1999) Voltage-independent autocrine modulation of L-type channels mediated by ATP, opioids and catecholamines in rat chromaffin cells. *Eur J Neurosci* 11:3574–3584
 58. Hess P, Lansman JB, Tsien RW (1984) Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* 311:538–544
 59. Hille B (1994) Modulation of ion channel function by G-protein coupled receptors. *Trends Neurosci* 17:531–536
 60. Hirano Y, Yoshinaga T, Murata M, Hiraoka WA (1999) Prepulse-induce mode 2 gating behavior with and without β -adrenergic stimulation in cardiac L-type Ca channels. *Am J Physiol* 276:C1388–C1345
 61. Hollins B, Ikeda SR (1996) Inward currents underlying action potentials in rat adrenal chromaffin cells. *J Neurophysiol* 76:1195–1211
 62. Hosey MM, Chien AJ, Puri TS (1996) Structure and regulation of L-type calcium channels – a current assessment of the properties and roles of channel subunits *Trends Cardiovasc Med* 6:265–273
 63. Hoshi T, Rothlein J, Smith SJ (1984) Facilitation of Ca^{2+} -channel currents in bovine adrenal chromaffin cells. *Proc Natl Acad Sci USA* 81:5871–5875
 64. Hoshi T, Smith SJ (1987) Large depolarization induces long openings of voltage-dependent calcium channels in adrenal chromaffin cells. *J Neurosci* 7:571–580
 65. Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature* 380:255–258
 66. Imaizumi T, Watanabe Y, Yoshida H (1991) Phosphorylation of G_i protein by cyclic AMP-dependent protein kinase inhibits its dissociation into α -subunits and $\beta\gamma$ -subunits by Mg^{2+} and GTP- γ -S. *Eur J Pharmacol* 201:189–194
 67. Johnson BD, Brousal JP, Peterson BZ, Gallombardo PA, Hockerman GH, Lai Y, Scheuer T, Catterall WA (1997) Modulation of the cloned skeletal muscle L-type Ca^{2+} -channel by anchored cAMP-dependent protein kinase. *J Neurosci* 17:1243–1255
 68. Kammermeier PJ, Jones SW (1998) Facilitation of L-type calcium current in thalamic neurons. *J Neurophysiol* 79:410–417
 69. Kamp TJ, Hu H, Marban E (2000) Voltage-dependent facilitation of cardiac L-type Ca channels expressed in HEK-293 cells requires β -subunit. *Am J Physiol* 278:H126–H136
 70. Kavalali ET, Plummer MR (1996) Multiple voltage-dependent mechanisms potentiate calcium channel activity in hippocampal neurons. *J Neurosci* 16:1072–1082
 71. Kavalali ET, Hwang KS, Plummer MR (1997) cAMP-dependent enhancement of dihydropyridine-sensitive calcium channel availability in hippocampal neurons. *J Neurosci* 17:5334–5348
 72. Kitamura N, Ohta T, Ito S, Nakazato Y (1998) Calcium channel current facilitation in porcine adrenal chromaffin cells. *Pflügers Arch* 435:781–788
 73. Kleppisch T, Ahnert-Hilger G, Gollasch M, Spicher K, Hescheler J, Schultz G, Rosenthal W (1992) Inhibition of voltage-dependent Ca^{2+} channels via α_2 -adrenergic and opioid receptors in cultured bovine adrenal chromaffin cells. *Pflügers Arch* 421:1331–137
 74. Kleppisch T, Pedersen K, Strübing C, Bosse-Doenecke E, Flockerzi V, Hofmann F, Hescheler J (1994) Double-pulse facilitation of smooth muscle α -subunit Ca^{2+} channels expressed in CHO cells. *EMBO J* 13:2502–2507
 75. Magnelli V, Baldelli P, Carbone E (1998) Antagonists-resistant calcium currents in rat embryo motoneurons. *Eur J Neurosci* 10:1810–1825
 76. Maroto R, López MG, Del Valle M, Naranjo JR, Mellstrom B, García AG (1995) Expression of the bovine striatal D2 receptor, but not the D1 receptor, in bovine adrenal medulla. *Mol Pharmacol* 47:40–50
 77. McDonough SI, Swartz KJ, Mintz IM, Boland LM, Bean BP (1996) Inhibition of calcium channels in rat central and peripheral neurons by ω -conotoxin-MV1C. *J Neurosci* 16:2612–2623
 78. Mei YA, Griffon N, Buquet C, Martres MP, Vaudry H, Schwartz J-C, Sokoloff P, Cazin L (1995) Activation of dopamine D_4 receptor inhibits an L-type calcium current in cerebellar granular cells. *Neuroscience* 68:107–116
 79. Mintz IM, Bean BP (1993) GABA $_B$ receptor inhibition of P-type Ca^{2+} channels in central neurons. *Neuron* 10:889–898
 80. Mintz IM, Bean BP (1993) Block of calcium channels in rat neurons by synthetic ω -Aga-IVA. *Neuropharmacology* 32:1161–1169
 81. Neely A, Wei X, Olcese R, Birbaumer L, Stefani E (1993) Potentiation of the β subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* 262:575–578
 82. Orts A, Orellana C, Canto T, Ceña V, González-García C, García AG (1987) Inhibition of adrenomedullary catecholamine release by propranolol isomers and clonidine involving mechanisms related to adrenoceptors. *Br J Pharmacol* 92:795–801
 83. Otsuguro K, Ohta T, Ito S, Nakazato Y (1996) Modulation of calcium current by ATP in guinea-pig adrenal chromaffin cells. *Pflügers Arch* 431:402–407
 84. Parramón M, González MP, Oset-Gasque MJ (1995) A reassessment of the modulatory role of cyclic AMP in catecholamine secretion by chromaffin cells. *Br J Pharmacol* 114:517–523
 85. Pietrobon D, Hess P (1990) Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature* 346:651–655
 86. Platano D, Qin N, Noceti F, Birbaumer L, Stefani E, Olcese R (2000) Expression of the α_2 - δ subunit interferes with prepulse facilitation in cardiac L-type calcium channels. *Biophys J* 78:2959–2972
 87. Pollo A, Lovallo M, Biancardi E, Sher E, Socci C, Carbone E (1993) Sensitivity to dihydropyridines, ω -conotoxins and noradrenaline reveals multiple high-voltage activated Ca^{2+} channels in rat insulinoma and human pancreatic β -cells. *Pflügers Arch* 423:462–471
 88. Przywara DA, Guo X, Angelilli ML, Wakade TD, Wakade AR (1996) A non-cholinergic transmitter, pituitary adenylate cyclase-activating polypeptide, utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. *J Biol Chem* 271:10545–10550
 89. Qin N, Platano D, Olcese R, Costantin JL, Stefani E, Birbaumer L (1998) Unique regulatory properties of the type 2a Ca^{2+} channel β subunit caused by palmitoylation. *Proc Natl Acad Sci USA* 95:4690–4695
 90. Reuter H (1983) Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* 301:569–574
 91. Scholz KP, Miller RJ (1991) GABA $_B$ receptor-mediated inhibition of Ca^{2+} currents and synaptic transmission in cultured rat hippocampal neurons. *J Physiol (Lond)* 444:669–686
 92. Schroeder TJ, Jankowski JA, Senyshyn J, Holz RW, Wightman RM (1994) Zones of exocytotic release on bovine adrenal medullary cells in culture. *J Biol Chem* 269:17215–17220
 93. Sculptoreanu A, Scheuer T, Catterall W (1993) Voltage-dependent potentiation of L-type Ca channels due to phosphorylation by cAMP-dependent protein kinase. *Nature* 364:240–243
 94. Sculptoreanu A, Rotman E, Takahashi M, Scheuer T, Catterall W (1993) Voltage-dependent potentiation of the activity of cardiac L-type calcium channel α_1 subunits due to phosphorylation by cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 90:10135–10139
 95. Sculptoreanu A, Figourov A, De Groat WC (1995) Voltage-dependent potentiation of neuronal L-type calcium channels due to state-dependent phosphorylation. *Am J Physiol* 269:C725–C732

96. Sher E, Cesare P, Codignola A, Clementi F, Tarroni P, Pollo A, Magnelli V, Carbone E (1996) Activation of δ -opioid receptors inhibits neuronal-like calcium channels and distal steps of Ca^{2+} -dependent secretion in human small-cell lung carcinoma cells. *J Neurosci* 16:3672–3684
97. Tiaho F, Richard S, Lory P, Nerbonne JM, Nargeot J (1990) Cyclic-AMP-dependent phosphorylation modulates the stereospecific activation of cardiac Ca channels by Bay K 8644. *Pflügers Arch* 417:58–66
98. Visentin S, Amiel P, Fruttero R, Boschi D, Roussel C, Giusta L, Carbone E, Gasco A (1999) Synthesis and voltage-clamp studies of methyl 1,4-dihydro-2,6-dimethyl-5-nitro-4-(benzofurazanyl)pyridine-3-carboxylate racemates and enantiomers and of their benzofuroxanyl analogues. *J Med Chem* 42:1422–1427
99. Winkler H, Westhead E (1980) The molecular organisation of adrenal chromaffin granules. *Neuroscience* 5:1803–1823
100. Xiao R-P, Ji X, Lakatta EG (1995) Functional coupling of the b_2 -adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol Pharmacol* 47:322–329
101. Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1996) Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. *Nature* 385:442–446
102. Zong X, Schreieck J, Mehrke G, Welling A, Schuster A, Bosse E, Flockerzi V, Hofmann F (1995) On the regulation of the expressed L-type calcium channel by cAMP-dependent phosphorylation. *Pflügers Arch* 430:340–347