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Direct and Remote Modulation of L-Channels in Chromaffin Cells

Distinct Actions on α_{1C} and α_{1D} Subunits?

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Abstract

Understanding precisely the functioning of voltage-gated Ca^{2+} channels and their modulation by signaling molecules will help clarifying the Ca^{2+}-dependent mechanisms controlling exocytosis in chromaffin cells. In recent years, we have learned more about the various pathways through which Ca^{2+} channels can be up- or down-modulated by hormones and neurotransmitters and how these changes may condition chromaffin cell activity and catecolamine release. Recently, the attention has been focused on the modulation of L-channels (Ca_V 1), which represent the major Ca^{2+} current component in rat and human chromaffin cells. L-channels are effectively inhibited by the released content of secretory granules or by applying mixtures of exogenous ATP, opioids, and adrenaline through the activation of receptor-coupled G proteins. This unusual inhibition persists in a wide range of potentials and results from a direct (membrane-delimited) interaction of G protein subunits with the L-channels co-localized in membrane microareas. Inhibition of L-channels can be reversed when the cAMP/PKA pathway is activated by membrane permeable cAMP analog or when cells are exposed to isoprenaline (remote action), suggesting the existence of parallel and opposite effects on L-channel gating by distinctly activated membrane autoreceptors.

Here, the authors review the molecular components underlying these two opposing signaling pathways and present new evidence supporting the presence of two L-channel types in rat chromaffin cells (α_{1C} and α_{1D}), which open new interesting issues concerning Ca^{2+}-channel modulation. In light of recent findings on the regulation of exocytosis by Ca^{2+}-channel modulation, the authors explore the possible role of L-channels in the autocontrol of catecholamine release.

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Introduction

Voltage-gated L-type Ca^{2+} channels are widely distributed in excitable cells. They include several subtypes containing the pore-forming α_{1S}, α_{1C}, α_{1D}, and α_{1F} subunits (Ca_v 1.1, Ca_v 1.2, Ca_v 1.3, and Ca_v 1.4). Members of the L-type channel family open upon membrane depolarization and represent one of the main pathways by which cytoplasmic Ca^{2+} levels can be regulated in neuronal and neuroendocrine cells *(1,2).* As the elevation of intracellular Ca^{2+} represents the triggering event of hormone secretion and cell differentiation *(3,4),* the precise characterization of L-channels functioning and their modulation is important for understanding key issues about neuroendocrine cells activity and neuronal development.

Modulation of Ca^{2+}-entry through voltage-gated L-channels may occur in various ways: 1) by down regulating or recruiting newly available channels; and 2) by inhibiting or facilitating the channel gating (*see* refs. *5–7* for reviews). L-channel gating modulation has received great attention in the last 20 yr and appears an effective system for controlling Ca^{2+} ions entering the cell. Among the many modulatory pathways, two appear of particular interest because of their autocrine nature: the G protein-dependent inhibition and the cAMP/PKA-mediated potentiation *(5–7).* In chromaffin cells, both pathways are activated by autoreleased neurotransmitter molecules and produce opposing effects of comparable entity *(8).* The inhibition is complete within few seconds and is mediated by PTX-sensitive G proteins coupled to P_{2y}-purinergic, μ/δ-opioidergic, α_2- and β_2-adrenoreceptors (ARs) *(9–14).* In contrast, the potentiation is selectively triggered by β_1-ARs and occurs slowly through the activation of a cAMP/PKA pathway, which may act at distant sites from receptors *(15,16).*

An open question relative to these opposing effects is whether they target two distinct L-channel types (α_{1C} and α_{1D}) or do they rather converge on the same α_1 subunit. Since chromaffin cells, like other neuroendocrine cells express variable densities of α_{1C} and α_{1D} subunits *(17–20),* it is intriguing to speculate that potentiation and inhibition may proceed in parallel on separated L-channels (Ca_v 1.2 or Ca_v 1.3). However, as the authors demonstrate here, the available data do not support this view. A second interesting issue is whether Ca^{2+} changes associated to L-channel modulation play a critical role in the control of exocytosis. Recent observations suggest that down- or up-modulation of L-currents do not always produce proportional effects on exocytosis, indicating that intracellular [Ca^{2+}] increases following Ca^{2+} channels activation are preliminary to secretion but may be either amplified *(21)* or depressed *(22)* by a downstream action on the secretory machinery. Besides shortly reviewing the molecular features of G protein-induced inhibition and cAMP-mediated potentiation of L-channels, the authors furnish new evidence supporting the existence of α_{1C} and α_{1D} subunits in rat chromaffin cells and discuss some recently uncovered peculiarity associated to L-channel modulation and its coupling to secretion.

Forms of L-Channel Gating Modulation

L-channel modulation is largely heterogenous and covers a broad spectrum of molecular mechanisms. A major subdivision should include the signaling pathways that are either voltage-dependent or voltage-independent. Among the first class should be mentioned: 1) the voltage-dependent facilitation producing L-current increases following strong and long-lasting prepulses described in cardiac, neuronal, and neuroendocrine cells *(23–25)* and; 2) the voltage-dependent and cAMP-mediated phosphorylation, capable of facilitating the L-channel expressed in skeletal muscle, neuronal, and neuroendocrine cells by a fast phosphorylation reaction favored by strong

depolarizations *(26–28)* and by the close proximity of PKA to the channel *(29).* Both modulatory pathways have been described in recent review articles *(6,7).* Here, the authors focus on the voltage-independent forms of neuroendocrine L-channel modulation that include the direct inhibition of L-channels by G protein-coupled receptors (GPCRs) and the cAMP-mediated potentiation that have autocrine origins and can be back-regulated by the material released during secretion.

Direct Inhibition of Neuroendocrine L-Channels by $G_{i,o}$ Proteins

Whole-Cell Recordings

Neuronal and neuroendocrine L-channels can be effectively inhibited by applied neurotransmitters through GPCR-mediated pathways. The action, however, is quite variable. In most cases the inhibition is V-independent. There is no delay of L-channel activation and the depression causes mostly a 20–60% inhibition of the current *(8,11,13,14,30–46).* There are, however, examples in which the neurotransmitter has no action *(47–49)* or the inhibition is even V-dependent, resembling closely that of N- and P/Q-channels (*see* ref. *5*). Despite this variability, the V-independent and GPCR-mediated inhibition of L-channels appears to be a widespread mechanism of Ca^{2+} signaling in neuronal and neuroendocrine cells; although attempts to mimic this functional property in heterologous systems has failed so far *(50–51).* In Table 1 a number of papers showing evidence for a V-independent GPCR-mediated inhibition of L-type Ca^{2+} currents in neurons and neuroendocrine cells is listed. Notice the number of GCPRs and neurotransmitters involved and the broad spectrum of cell preparations: sensory and central neurons, as well as β-pancreatic, chromaffin, adrenal glomerulosa, melanotrophic, and pituitary cells.

In the chromaffin cells of adrenal medulla, the neurotransmitter-mediated inhibition of L-channels is mainly V-independent. The process is triggered by the same neurotransmitters released by the chromaffin granules (ATP, opioids, and catecholamines) and produces a scaling down of the current amplitude *(13).* Figure 1A shows a representative action of ATP, μ/δ opioid agonists (DAMGO and DPDPE), adrenaline (A) and noradrenaline (NA) when applied directly on a rat chromaffin cell (RCC) pretreated with ω-CTx-GVIA and ω-Aga-IVA to minimize N- and P/Q-currents contribution (exogenous inhibition). In Fig. 1B, the control current recorded from a toxin-treated RCC is strongly inhibited by mixtures of ATP and opioid agonists. The inhibition causes a size reduction with no changes to the activation timecourse and a facilitatory prepulse to +100 mV is unable to recover the inhibition. Notice that, in bovine chromaffin cells (BCCs) *(10)* and RCCs *(13)* the L-current changes its amplitude depending on the conditions of cell superfusion. In stop–flow conditions the current is about half of the size recorded during rapid flow, which quickly washes off the secreted material. A facilitatory prepulse to +100 mV is ineffective in recovering part of the depression. This indicates that L-channel inhibition by neurotransmitters is autocrine and can act as a negative feedback to control Ca^{2+} fluxes and neurotransmitter release in secretory cells (endogenous inhibition). The involvement of G proteins in the endogenous and exogenous inhibition of L-channels is supported by data showing that intracellular perfusion of GDP-β-S, cell pretreatment with PTX and application of autoreceptor antagonists largely prevent the effect *(13).*

Single-Channel Recordings

An important issue concerning the V-independent autocrine inhibition of L-channels is whether the mechanism requires a diffusable second messenger or is direct (membrane-delimited) on the target channel. A direct action of $G_{i,o}$ proteins on L-channels is already suggested by the fast onset and offset of the

Table 1
Reported Evidence of V-Independent L-Type Channel Inhibition by Neurotransmitters

Cell type	% block	Ca^{2+} or Ba^{2+}	GPCR or agonist	PTX-sensitive	Kinetics of inhibition	V-independence[i]	Reference
Rat hippocampal neurons	20	Ba^{2+}	$GABA_B$	yes	fast	time course	*30*
Undifferentiated PC12 cells	30–40	Ba^{2+}	α-AR, muscarinic	yes	fast	time course	*31*
Rat pituitary GH3 cells	25–30	Ba^{2+}	somatostatin, muscarinic	yes	fast	time course	*32*
Rat insulinoma RINm5F cells	25	Ba^{2+}	noradrenaline	yes	fast	double pulse	*33*
Human pancreatic β-cells	25	Ba^{2+}	noradrenaline	–	fast	double pulse	*33*
Mouse cerebellar neurons	marked	Ba^{2+}	GTP-γ-S	yes	fast	time course	*34*
Mouse cerebellar granules	61 (NPo)	Ba^{2+}	$mGluR_2$ $mGluR_3$	yes	membrane-delimited	time course	*35*
Rat cerebellum granules	40	Ba^{2+}	$GABA_B$	yes	fast	I-V curve	*36*
Rat cerebellum granules	75	Ba^{2+}	D_4	yes	fast	time course	*37*
Bovine chromaffin cells	70	Ba^{2+}	μ/δ-opioids	yes	fast	double-pulse	*11**
Rat pituitary AtT-20 cells	30	Ca^{2+}	sst_2, sst_5	yes	fast	I-V curve	*38*
Mouse pancreatic β-cells	35	Ca^{2+}	muscarinic	GDP-β-S	fast	I-V curve	*39*
Rat neurohypophysis	64	Ca^{2+}	k-opioids	–	fast	time course	*40*
Rat sensory neurons	54	Ba^{2+}	dopamine	–	fast	double-pulse	*41*
Lamprey spinal motoneurons	25	Ca^{2+}	dopamine	GDP-β-S	fast	double-pulse	*42*
Bovine glomerulosa cells	63	Ba^{2+}	AT_1, angiotensin II	yes	fast	I-V curve	*43*
Rat chromaffin cells	50	Ba^{2+}	α-, β-AR, δ-opioids, P_{2y}	yes	fast	double-pulse	*13*
Rat sensory neurons	55	Ba^{2+}	δ-opioids	–	fast	time course	*44*
Salamander rod photoreceptors	33	Ba^{2+}	sst_{2A}	yes	fast	I-V curve	*45*
Bovine chromaffin cells	50 (Po)	Ba^{2+}	δ-opioids, P_{2y}	yes	membrane-delimited	double-pulse	*14***
Rat melanotrophs	75	Ba^{2+}	dopamine	–	slow	time course	*46*
Rat chromaffin cells	35	Ca^{2+}	β_2-ARs	yes	fast	I-V curve	*8*

In the order are indicated: the cell type, the percentage of L-channel inhibition, the charge carriers (Ca^{2+} or Ba^{2+}), the type of G protein-coupled receptors (GPCRs) or agonists involved, the kinetics of the action (whether fast or slow in whole-cell recording or membrane-delimited in single-channel recording) and whether has been used a double-pulse protocol, an I-V curve or the time course of activation to test the V-independence. Papers are listed in chronological order.

* The inhibition is referred to a depression of the open probability (NPo) in multichannel patches

** The inhibition is referred to a depression of the open probability (Po) in single channel patches

[i] Voltage-independence was established by either an analysis of the double-pulse protocol, I-V curve or time course of L-current activation

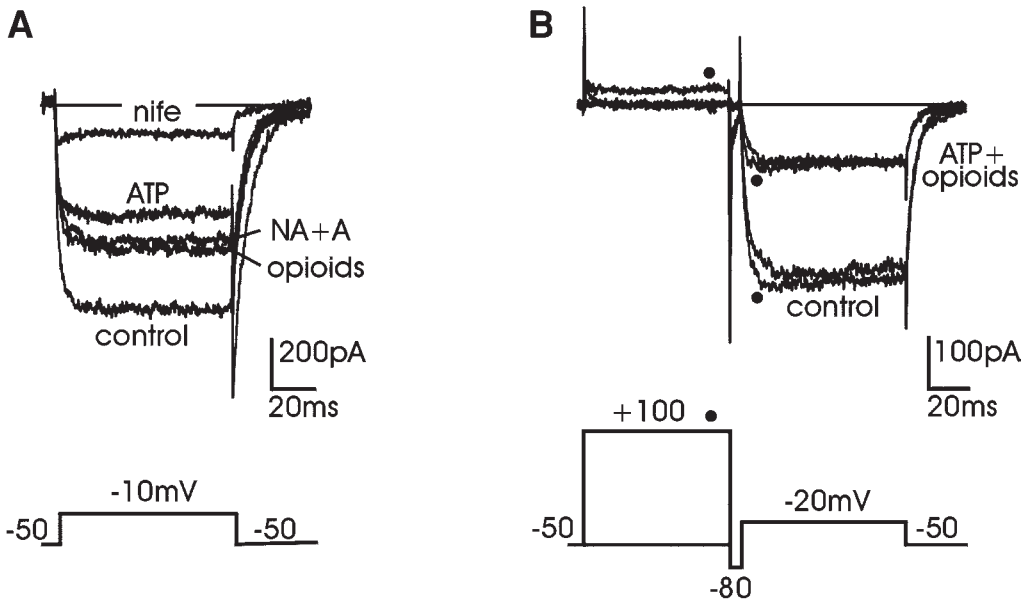

Fig. 1. Voltage-independent inhibition of L-type currents by ATP, opioids, adrenaline (A), and noradrenaline (NA) in RCCs. Cells were pre-incubated with 1 μ*M* ω-CTx-GVIA and 2 μ*M* ω-Aga-IVA to remove most of the available N- and P/Q-type channels. 1 μ*M* Bay K 8644 was added to the bath to enhance the size of the currents in 5 m*M* Ba^{2+}. In both cases, 5 μ*M* nifedipine blocked most of the inward Ba^{2+} current. Pulse protocols are indicated below the recordings. (Modified from ref. *13*.)

inhibition during rapid application and withdrawal of neurotransmitters to RCCs *(13)*. The onset of the ATP-induced inhibition is rapid (τ_{on} 0.75 s) and the offset complete within 20 s, i.e., comparable to that of neurotransmitters-induced inhibition of N-type channels *(52,53)*, which is known to be membrane-delimited (*see* ref. *54*). However, the most convincing evidence for a direct action comes from single-channel studies in which the $G_{i,o}$ protein-dependent inhibition of L-channels is shown to be autocrine and fully defined in cell-attached patches *(12,14)*.

Figure 2 shows the main characteristics of this modulation viewed at the single-channel level under favorable conditions for measuring L-channels (100 m*M* Ba^{2+}, 10 μ*M* ω-CTx-MVIIC and 5 μ*M* Bay K 8644). In control conditions (with ATP and opioids released locally inside the pipet), openings are rare and separated by long closures during depolarizations of 320 ms to +10 mV and the number of null sweeps is relatively high (33%) *(14)*. The probability of opening is low (P_O 0.27) despite the presence of Bay K 8644 in the pipet. The activity of single L-channels, however, is significantly enhanced if opioidergic and purinergic antagonists such as naloxone and suramin are included in the pipet (Fig. 2, bottom), or if the cells are pretreated with PTX to prevent the activation of inhibitory $G_{i,o}$ proteins *(14)*. Channel activity is clearly augmented under these conditions. With the antagonists, P_O increases and the amplitude of averaged currents are nearly doubled while the number of nulls is approximately halved. Analysis of open and closed time distributions shows that the enhanced P_O is mostly due to a shortening of mean closed times rather than a prolongation of mean open times, suggesting higher frequency of openings rather than increased open durations.

The results of Fig. 2 allow some interesting conclusions. First, as for the N- and P/Q-channels

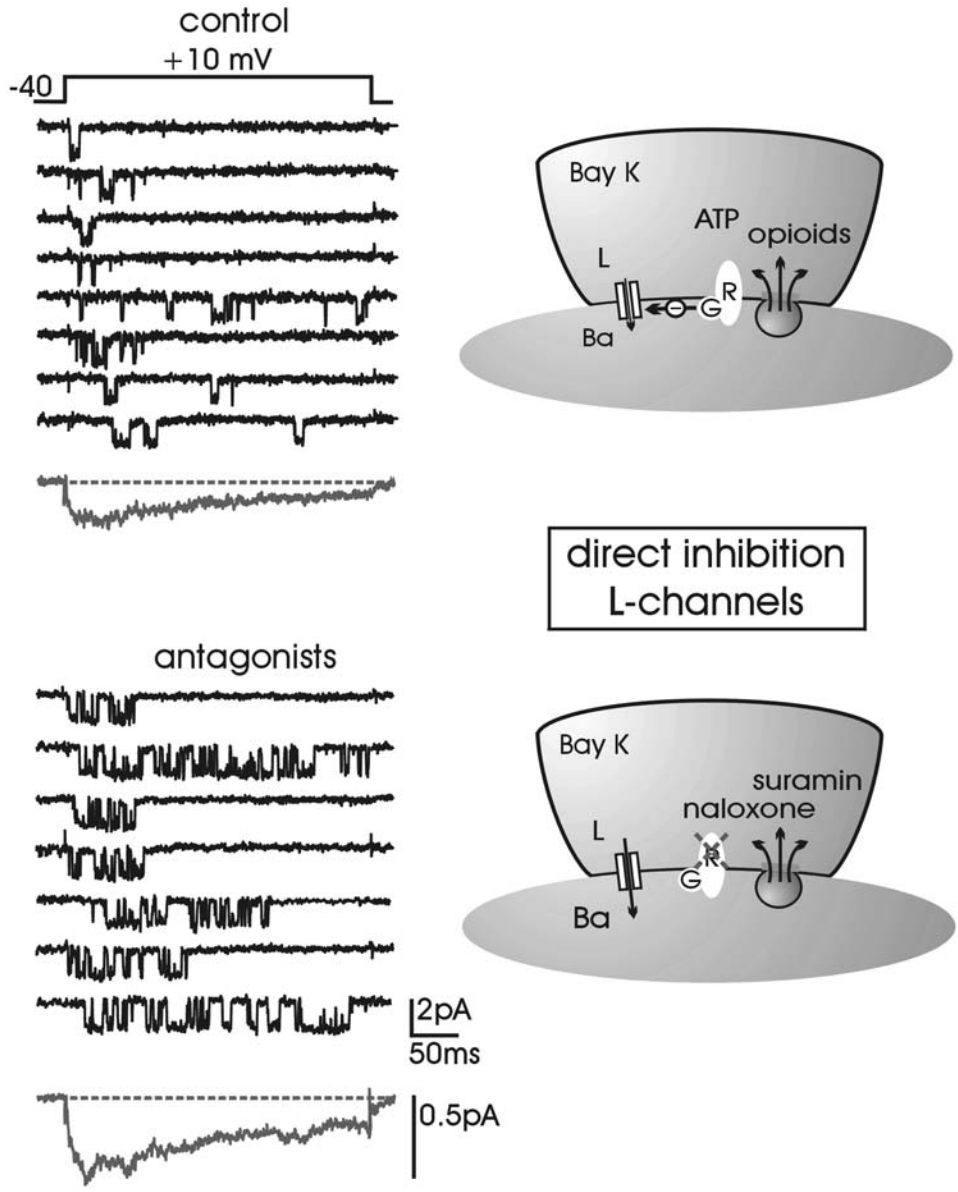

Fig. 2. L-channel activity in cell-attached patches of BCCs is enhanced by addition of purinergic and opioidergic antagonists to the patch-pipet (direct autocrine inhibition). The single channel recordings were performed at +10 mV in control condition (top) with 100 m*M* $BaCl_2$, 5 µ*M* Bay K 8644 and 10 µ*M* ω-CTx-MVIIC in the pipet and in the presence of 100 µ*M* suramin and 10 µ*M* naloxone (bottom). Averaged currents are shown at the bottom of each panel. (Modified from ref. *14*.)

(12), the L-channel activity in control patches is strongly conditioned by the endogenous inhibition due to the material released inside the pipet during patch stimulation with Ba^{2+} (local release). Second, the inhibition of L-channels in BCCs is fully defined in membrane patches of approx 1 µm diameter, suggesting no requirement for diffusable second messengers. Third, the local

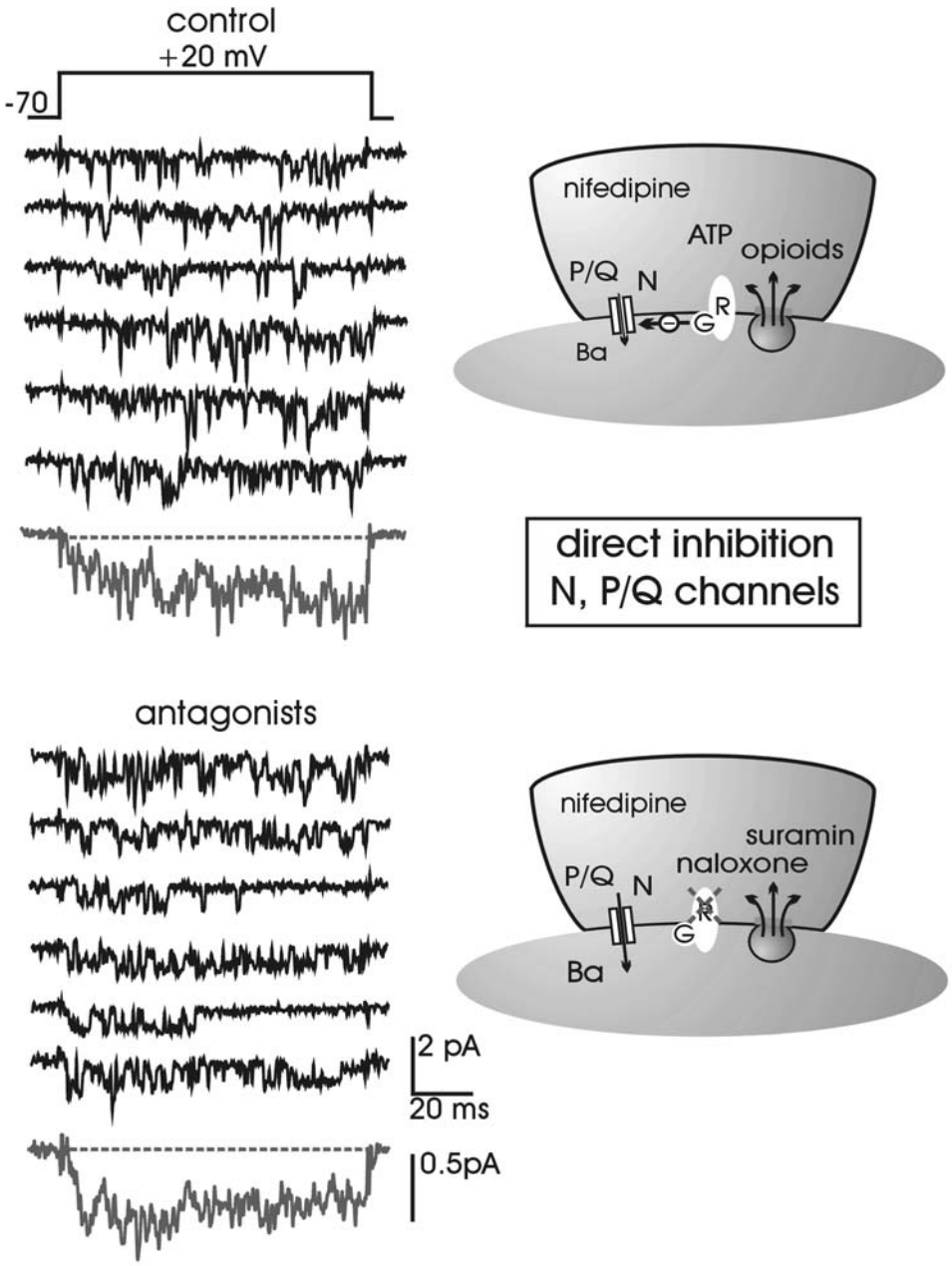

Fig. 3. First openings of N and P/Q-channels in cell-attached patches of BCCs are accelerated by addition of purinergic and opioidergic antagonists to the patch-pipet (direct autocrine inhibition). The single-channel recordings were performed at +20 mV in control condition (top) with 100 m*M* $BaCl_2$, 5 μ*M* nifedipine in the pipet and in the presence of 100 μ*M* suramin and 10 μ*M* naloxone (bottom). Averaged currents are shown at the bottom of each panel (Modified from ref. *12*.)

inhibition induced by released neurotransmitters does not alter the channel activation kinetics, neither delays channel openings. Thus, unlike N- and P/Q-channels *(55–57)*, there is no time- and V-dependence associated to channel gating. This is confirmed by the absence of any voltage-dependent facilitation following double pulse protocols in the presence of endogenous or exogenous neurotransmitters *(7,14)*. For comparison, Fig. 3 shows the effects of released neurotransmitters (ATP and opioids) on the activation kinetics of N- and P/Q-channels in cell-attached patches of BCCs in the presence of 5 μ*M* nifedipine *(12)*. Control-averaged currents activate very slowly owing to the time- and voltage-dependent inhibition induced by the $G_{i,o}$ protein subunits activated by membrane autoreceptors *(52–57)* whereas activation is fast and complete in the presence of P_{2y}-purinergic and μ/δ-opioidergic antagonists.

Remote Potentiation of L-channels by cAMP/PKA Signaling

Cardiac α_{1C} L-channel activity can be effectively potentiated by β-adrenergic stimulation, direct adenylate cyclase activation or application of membrane diffusable forms of cAMP *(58–60)*. All three pathways induce elevations of intracellular cAMP, activation of PKA, and phosphorylation of Ca^{2+} channels even if the substrates responsible for the potentiating action are not yet fully defined *(61,62)*. 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 which express high densities of L-channels and β_1-AR. Elevation of intracellular cAMP produces a marked enhancement of channel open probability (P_O) and the recruitment of newly functioning L-channels, with a resulting three- to fourfold increase of Ca^{2+} current amplitude *(58,63)*. The higher P_O value 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 effects of cAMP resembles the potentiating action of Ca^{2+}-channel agonists *(64)*, although the two mechanisms appear additive in enhancing the mean P_O of the channel *(65)*.

Despite the overwhelming literature supporting the existence of a cAMP-mediated potentiation of native α_{1C} channel gating, the regulation of cloned class C channels expressed in heterologous systems has not yet been reproduced. In some cases, the potentiating effect was either smaller than observed in cardiac myocytes *(66–68)* or even absent *(69,70)*. The reason could depend on the critical role of various molecular determinants. The first is the amino acid sequence of the α_{1C} subunits, which should contain the consensus site for PKA phosphorylation identified in the serine residue Ser 1928 in the distal part of the C-terminal domain *(62)*. A second requirement is possibly the presence of a specific A-kinase anchoring protein (AKAP) not yet identified that should anchor PKA close to the α_{1C} channels, favoring their phosphorylation and a weak stimulation of the channel *(67)*. The third one is the method chosen to record Ca^{2+} currents, which should not be invasive and is capable of preserving the intracellular environment medium (cell-attached or perforated-patch vs whole-cell recordings). This could be particularly relevant when dealing with modulations involving catalytic subunits and diffusable second messengers (*see* refs. *7,8,71*).

The cAMP-mediated channel gating potentiation is not confined to cardiac α_{1C} L-type channels. Effective cAMP/PKA-mediated phosphorylations leading to an increased L-type current have been reported in central neurons *(72)* and mouse pancreatic β-cells *(73,74)*. L-channels of BCCs and RCCs also undergo a cAMP/PKA-mediated potentiation. In BCCs, application of cAMP causes a markedly increased open probability which results in an increased L-channel activity mainly due to a decrease of channel closed times and number

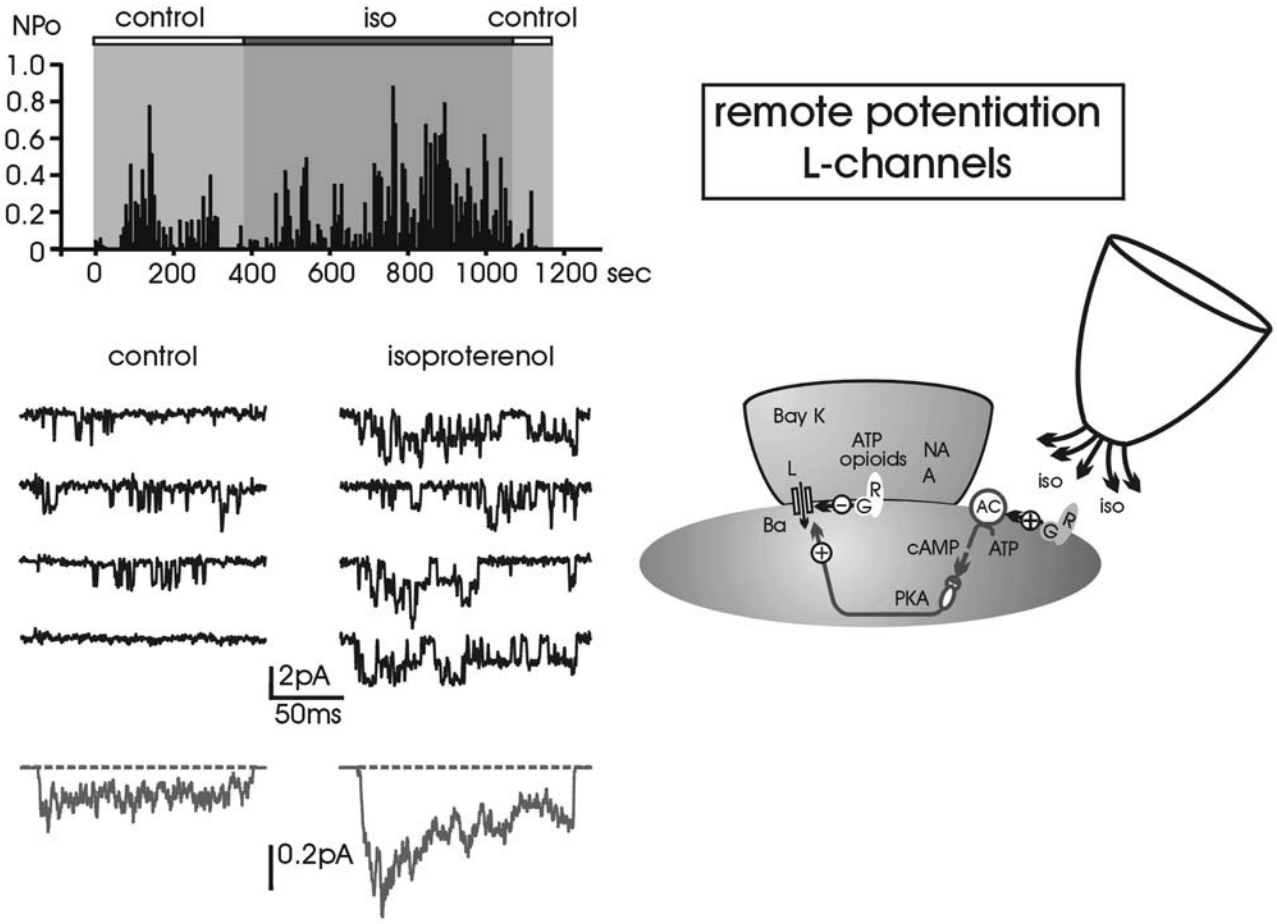

Fig. 4. Remote application of isoprenaline up-regulates L-channel activity in a RCC cell-attached patch. On the top are plotted the NPo values at +10 mV recorded sweep by sweep when the cell was sequentially perfused with a control solution, isoprenaline (10 μ*M*) and control again. Increment of NPo after approx 3–4 min from bath addition of isoprenaline, which is the time required for the cAMP/PKA mediated phosphorylation of L-channels to occur. (Modified from ref. *7*.)

of null sweeps rather than an increase of mean open times *(14)*. The cAMP-mediated potentiation is prevented by the PKA selective inhibitor H89 and proceeds regardless of the presence of the $G_{i,o}$ protein-mediated inhibition. In RCCs the cAMP/PKA-induced potentiation of L-channels is mediated by β_1-adrenergic receptors (β_1-ARs), which are capable of increasing Ca^{2+} currents and thus potentiating catecholamine release in an autocrine manner. β_1-ARs stimulation in RCCs *(7,8)* possess all the prerequisites of the remote action induced by the cAMP/PKA signaling pathway described in cardiac cells: 1) is mediated by isoprenaline and removed by propranolol; 2) is selective for L-type channels and prevented by the PKA selective inhibitor H89; 3) requires several minutes to reach maximal steady-state conditions; 4) is voltage-independent; and 5) is induced by application of isoprenaline outside the patch–pipet recording areas in cell-attached conditions (*see* Fig. 4). The presence of a cAMP/PKA-mediated pathway modulating the L-type channels of chromaffin cells represents a unique example of

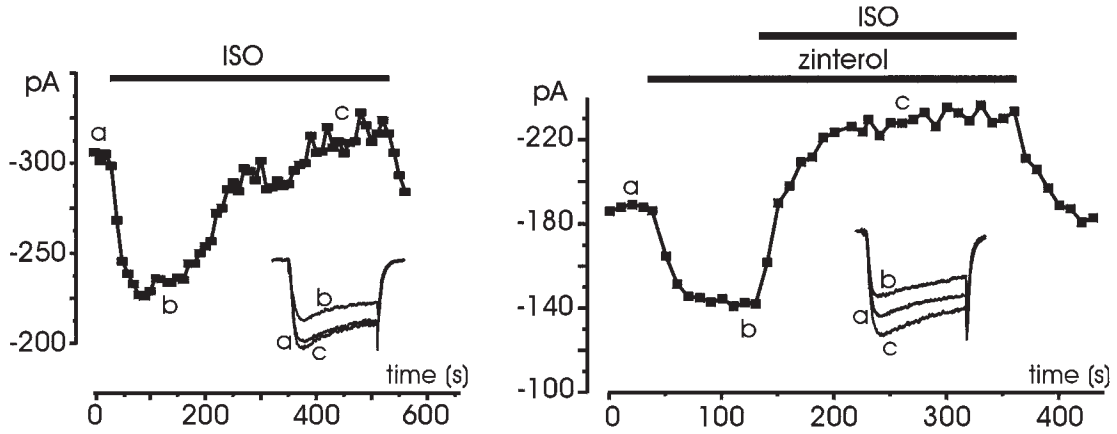

Fig. 5. Sequential inhibition and potentiation of L-type Ca^{2+} currents during β_2- and β_1-ARs stimulation in RCCs. On the left, isoprenaline alone (1 μ*M*) caused the rapid inhibition and slow recovery of the current amplitude. On the right, the slow action of isoprenaline followed the fast inhibition induced by zinterol (selective β_2-ARs agonist; 1 μ*M*). The symbols are peak current amplitudes measured on step depolarization to +10 mV repeated every 10 s (Vh –40 mV). The insets show the original recordings taken at the time indicated. (Modified from ref. *8*.)

positive feedback signaling involved in the autocontrol of neurotransmitter release. However, the β-AR modulation of L-channels in rat chromaffin cells possesses an even more striking peculiarity, which is illustrated below.

The β_1- and β_2-ARs Modulation of L-Channels in RCCs: An Example of Direct and Remote Signaling Pathways Acting in Parallel

The $G_{i,o}$ protein-dependent inhibition of L-channels in RCCs is not limited to opioidergic, purinergic, and α-adrenergic autoreceptors. There is in fact evidence also for the involvement of β-ARs. β-ARs and α-ARs are both implicated in the autocrine inhibition of L-channels, which is mostly evident when L-currents are recorded from individual chromaffin cells under stop–flow conditions, i.e., from RCCs bathed in a medium containing the granule content released by surrounding cells (*see* Fig. 8 in ref. *13*). Given this, the question is whether there is any rationale for the presence of β-ARs, which can up- or down-modulate the major Ca^{2+} current component controlling neurotransmitter release in RCCs. The answer comes from recent findings in which the authors show that RCCs express two distinct β_1- and β_2-AR activated signaling pathways *(8)*. The β_1-AR cascade acts by selectively up-regulating the L-channel through a PKA-mediated pathway and develops slowly due to its diffusive characteristics. On the contrary, the β_2-AR signaling is fast and primarily coupled to PTX-sensitive G proteins. Figure 5 (to left) shows a representative case in which a RCC responds to ISO stimulation with rapid inhibition of Ca^{2+} currents followed by a slow potentiation. The final balance is a slight increase of Ca^{2+} currents, which allows the cell to elevate cAMP concentration, with little increases of Ca^{2+} fluxes. This is quite different from the β-AR stimulation of cardiac cells in which the cAMP/PKA stimulation is accompanied by an amplification of Ca^{2+} fluxes required for increasing the strength of cell contraction during sympathetic stimulation *(75)*. In the case of chromaffin cells, the increased levels of cAMP are likely to be associated to an enhanced exo-

cytotic activity *(21)* and to the recruitment of T-type and Na^+ TTX-resistant channels *(76)* which may facilitate action potential generation during RCCs activity (*see* below).

An interesting aspect of the β-ARs modulation in RCCs is the peculiar role of β_2-ARs, which are directly coupled to an inhibitory PTX-sensitive G protein pathway and are unable to produce L-currents potentiation through the activation of adenylate cyclase, as in cardiac myocytes *(77).* Figure 5 (to right) shows that zinterol (a β_2-AR selective agonist) does not produce the slow stimulatory effect of isoprenaline, but rather causes a fast inhibition of Ca^{2+} currents *(8).* Indeed, the sequential application of zinterol and isoprenaline effectively mimics the effects of isoprenaline alone, indicating that complete activation of β_2-ARs produces an inhibitory effect followed by a slow potentiation mediated by β_1-ARs. β_2-ARs activation through zinterol has fast onset (τ_{on} 6.5 s) and slow offset (τ_{off} 38.1 s) when applied for periods longer than 5 min, indicating slow unbinding from the receptor site after prolonged exposure *(77).* In addition, the action of β_2-AR differs from that of β_1-AR mainly in the mode of action. β_2-ARs appear colocalized with G proteins, adenylate cyclase, PKA-anchoring proteins and phosphatases, ensuring rapid activation or deactivation of specific signaling pathways (direct action) *(15,78–83).* To the contrary, β_1-ARs stimulation preserves most of the prerequisites of β_1-AR stimulation of L-channels in ventricular myocytes: it involves diffusable second messengers (cAMP and PKA), which phosphorylate the L-type Ca^{2+} channel (remote action).

An unresolved issue of the dual L-channel modulation is whether the inhibition and potentiation act on the same effector molecule (α_1 subunit) or proceeds toward distinct targets. According to the available electrophysiological data, the inhibition through PTX-sensitive $G_{i,o}$ proteins and the potentiation mediated by cAMP or isoprenaline seem to converge on a single class of L-channels, possibly the α_{1D} *(7,8,14).* However, an increasing amount of evidence suggests that chromaffin cells, as well as neurons and other neuroendocrine cells, express also class C channels, making the overall issue open to any possible interpretation. In the following paragraphs, the authors review all the evidence in favor of the existence of α_{1C} and α_{1D} in neuroendocrine tissues and attempt some conclusion concerning their role as possible targets of the two distinct signaling pathways.

The Neuronal and Neuroendocrine α_1-Subunits of L-Channels

α_{1C} and α_{1D} in Neuronal, Pancreatic, and Pituitary Cells

In central neurons, L-type currents are carried through α_{1D} and α_{1C} subunits possessing strong homologies with the cardiac α_{1C} and skeletal muscle α_{1S} Ca^{2+} channel subunits *(84–87).* Both L-channel subtypes are mainly localized in different regions of neuronal cell bodies and proximal dendrites *(88).* The two classes are also expressed in reactive astrocytes, but their degree of expression changes markedly during ischemic injury *(89,90).*

The two channel types are also effectively expressed in neuroendocrine tissues. In insulin secreting cells, most of the reports point to the presence of the α_{1D} subunit but there is also clear evidence for the presence of the α_{1C} isoform as well. A class D channel has first been cloned from human pancreatic β-cells *(91)* and found expressed in both rat pancreatic β-cells *(92)* and INS-1 cell line *(93).* Insulin secreting RINm5F cells possess two α_{1D} isoforms of different aminoacids length: a long one (2203 aa) with 95% identity to the human α_{1D} isoform and a short one (1668 aa) with high homology to the carboxyl-truncated forms of rat brain and HIT cells α_{1D} subunits. Interestingly, RINmF5 cells express also two α_{1C} subunits: a short form (199 kDa), which is the major constituent of the (+)-[3H]isradipine-labeled L-type channels immunoprecipitated by α_{1C} antibodies and a long form (240 kDa) which may serve as substrate for cAMP-dependent protein kinase

phosphorylation *(94).* The existence of α_{1D} and α_{1C} channels in RINm5F cells explains also the variability of the voltage range of activation of L-currents which occurs either at very negative *(95,96)* or at somewhat more positive voltages *(33),* possibly depending on the proportion of the two channel classes expressed under different cell-culture conditions. In most cases, however, the biophysical evidence supports a clear predominance of the α_{1D} subunit. Usually, α_{1D} subunits give rise to Ca^{2+} currents characterized by a markedly negative threshold of voltage activation (–60 to –50 mV in 10 mM Ba^{2+}), which is so negative that it can be confused with that of low-threshold T-type channels (*see* ref. *97*). At variance with rat and humans, mouse pancreatic β-cells express only class C channels *(98).* In fact, β-cells of control and α_{1D} knockout mice display similar L-currents, suggesting that α_{1D} contributes little to the Ca^{2+} currents of mouse β-cells *(*but *see ref. 99).*

α_{1C} and α_{1D} have also been found in rat pituitary GH3 cells *(100)* and rat adrenal glomerulosa cells *(93)* and there is now clear evidence for the expression of α_{1D} in mouse sinoatrial nodes *(101,102).* The presence of α_{1D} in these cells appears conditional for setting the pace maker current responsible for the slow depolarization of sinoatrial myocytes.

Identification of α_{1C} and α_{1D} Subunits in Chromaffin Cells of Different Species

α_{1C} and α_{1D} are likely to be both expressed in chromaffin cells, but few reports support this view. The clearest evidence for the presence of both subunits in these cells derives from experiments performed in the bovine species, where the presence of α_{1D} mRNA in cultured cells *(18)* and its localization in specific regions of the adrenal medulla has been established *(19).* The only data in favor of the presence of α_{1C} are limited to one report showing that BCCs express an epitope specific for this subunit *(17).* Support to the presence of α_{1C} and α_{1D} in chromaffin cells comes also from the observation that the two subunits are present in undifferentiated rat PC12 cells. In these cells, NGF decreases the expression of α_{1C} more rapidly than α_{1D} *(20).*

Given the importance of establishing the existence of the two L-channel subtypes in RCCs, the authors investigated the presence of α_{1C} and α_{1D} subunits in these cells by using reverse transcription followed by PCR amplification (RT-PCR) of total RNA obtained from a population of freshly isolated RCCs. Two pairs of specific and powerful primers were designed from the nucleotide sequence of the rat brain α_{1C} and α_{1D} subunits (accession numbers P22002 and P27732, respectively). To rule out a potential contamination of genomic DNA, forward and reverse primers were designed from different exons. In addition, control RT-PCR experiments performed in the absence of AMV-reverse-transcriptase did not yield any fragment. Two PCR bands corresponding to the predicted sizes of α_{1C} (356 bp) and α_{1D} (367 bp) subunits were visualized upon agarose gel electrophoresis of the amplified products (Fig. 6, left). Moreover, single-cell RT-PCR analysis of the α_1-subunit RNA expression determined in individual RCCs (*see* Fig. 6, left) indicated that α_{1C} and α_{1D} transcripts were also expressed at the single-cell level. For these experiments, RNAs from individual cells were harvested into a patch pipet under RNase-free conditions using the same primers as above. As control, the authors tested the subunit expression in single RINm5F cells, which express both types of α_1 subunits *(92,94,103).* Figure 6 (right) shows a representative example of the results obtained in individual RCCs (out of 12) or RINm5F cells (out of 5); the authors observed two single bands of the predicted sizes for α_{1C} and α_{1D} subunits in both cell types. Although the intensity of the PCR bands detected in individual RCCs were variable, the α_{1C} and α_{1D} subunit mRNAs were clearly detectable in all the 12 individual cells tested. Control experiments sampling the buffer surrounding the cells always gave negative results. Further support to the presence of α_{1C} and α_{1D} subunits comes from the immunocytochemistry data of Fig. 7A. Immunoreactivity for the two L-type α_1 subunits was observed in all

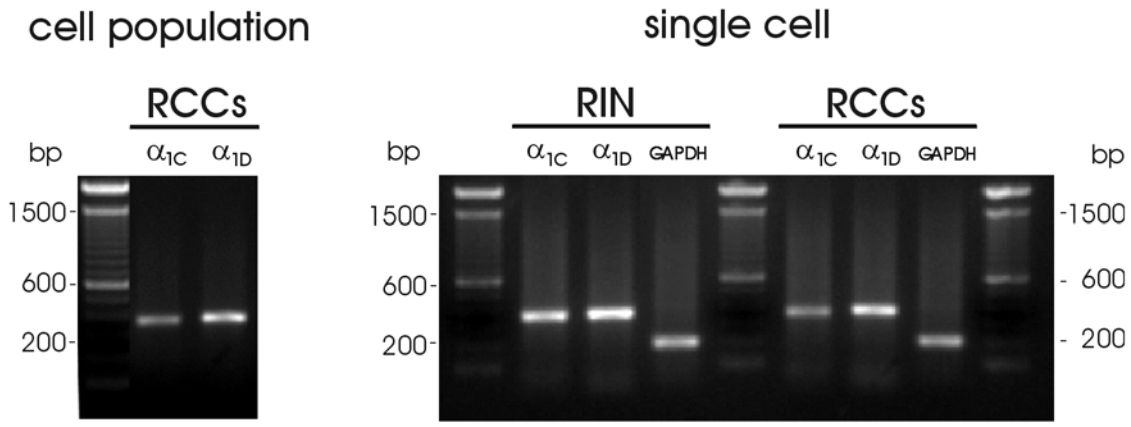

Fig. 6. Identification of transcripts for α_{1C} and α_{1D} subunits in RCCs and insulin-secreting RINm5F cells. (Left) Agarose gel electrophoresis of the α_1 subunits expressed in a population of RCC freshly isolated from adult rat adrenal glands as previously described *(13).* Total RNA extraction from these cells, cDNA synthesis and PCR reaction were performed as described previously *(18).* The following set of primers (from 5′ to 3′) were used to amplify the Ca^{2+} channel subunits: [α_{1C}] forward: GACCGGGGAGGACTGGAA, reverse: TGATCTTG-GTAGTGGGCG; [α_{1D}] forward: TGAGACACAGACCAAGCGAAGC, reverse: GTTGTCACTGTTGGCTATCTGG. After PCR amplification, 10 µL of each individual reaction sample was run on a 1.5% agarose gel and stained with ethidium bromide using a 100 bp DNA ladder as molecular weight marker (GIBCO/BRL). Two bands, corresponding to the amplified products for α_{1C} (356 bp) and α_{1D} (367 bp) subunits, were identified in the gel. The identity of these products was further confirmed by subsequent sequencing using the Big Dye method and the automatic sequencer ABI PRISM 377 (Perkin Elmer). (Right) Agarose gel electrophoresis of the amplified cDNA fragments corresponding to the α_{1C} and α_{1D}. The single-cell RT-PCR analysis of the mRNAs corresponding to both α_1 subunits was performed following protocols described elsewhere *(128,129),* including the overnight RT reaction followed by two rounds of PCRs, using the same α_1 primers as above. As internal control of the assay, we used the following pair of primers based in the nucleotide sequence of the rat brain GAPDH (accession number P04797): forward CACCACAAAGTATTAAAGAACAGG, reverse TTCCTTCTCTTACTACCAATTCC. A product of 200 bp corresponding to the GAPDH mRNA, along with the two α_1 cDNA amplified fragments, were visualized in the gel. Molecular weights of the standards are shown on the left and right sides of the figure.

visualized cells, revealing coexpression. Using a similar immunocytochemistry approach, the authors also tested the presence of the two classes of L channels in insulin secreting RINm5F cells. In Fig. 7B, the fluorescence images derived from populations of RINm5F cells, stained five days after plating, show immunoreactivity for both α_{1D} and α_{1C} subunits.

Biophysical Characteristics of α_{1C} and α_{1D} in Neuroendocrine Tissues

The existence of two types of L-channels in chromaffin cells in RCCs, suggests some caution in interpreting data on L-channel modulation. If RCCs express a predominant class of L-channels, it is reasonable to expect that the biophysical properties of L-channel recordings reflect this majority. Indeed, the two channel types (α_{1C} and α_{1D}) cannot be easily distinguished on the basis of their affinity for DHP—agonists and antagonists, and neither for their single-channel conductance, mean open and mean closed times. The only parameter that appears significantly different between the two channel types is the voltage range of activation that is consistently shifted by 20–25 mV towards more negative potentials for the α_{1D} subunit. This occurs in heterologous systems and cultured cells *(104,105)* (but *see* ref. *50*). Clear examples

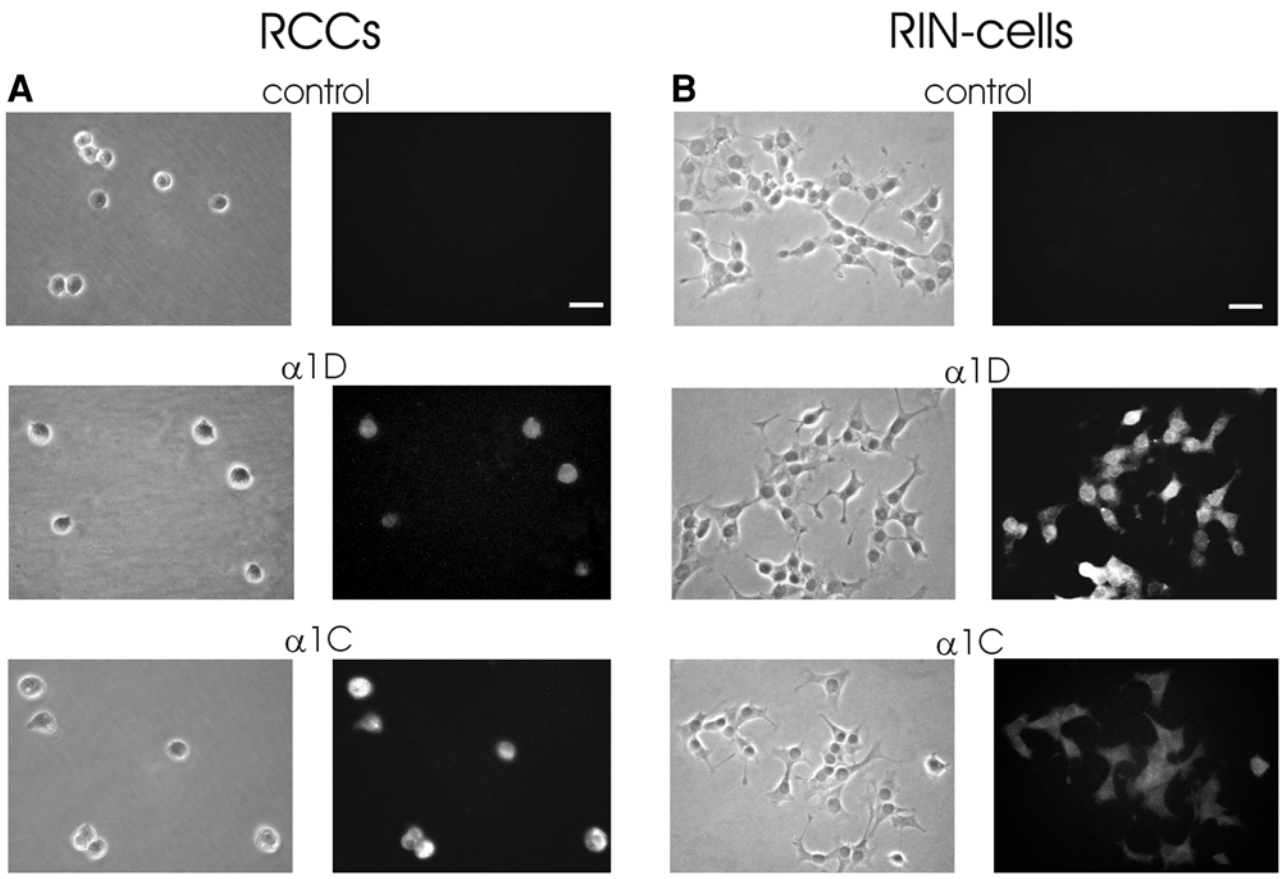

Fig. 7. Immunocytochemistry reveals co-expression of α_{1C} and α_{1D} subunits in rat chromaffin cells and RINm5F cells. Fluorescence images showed immunoreactivity for the two subunits decoding for L-type Ca^{2+} channels, α_{1C} and α_{1D}, in the totality of the RINm5F and chromaffin cells, clearly revealing their coexpression. The antibodies directed against L-type α_1 subunits, here used, have previously been characterized in terms of specificity and immunoreactivity and have proved to be well-suited for immunocytochemistry *(130,131).* In a number of different control experiments it was observed that omission of primary antibody yielded only background labeling as shown on the top line pictures of panels A and B. Marked specificity of the two primary antibodies was also proved by observing that RCCs and RINm5F cells lacking L-type Ca^{2+} currents showed always negative immunoreactivity, a few hours after enzymatic dissociation. Scale bars represent 20 μm for both panel A and B. **(A)** RCCs stained 1–3 d after plating, and **(B)** RINm5F insulinoma cells line showed immunoreactivity for both α_{1C} and α_{1D} subunits. Cells were grown in culture for 2–10 d on four wells Petri dishes, and were accurately washed in a buffer made up of PBS containing BSA 1%, NaN_3 0.05% and sucrose 5%. Fixation was performed in methanol 2% for 5 min at –20°C or in PAF 4% for 5 min. After many attempts, methanol was preferred to PAF for its ability to significantly reduce autofluorescence background caused by catecholamine content of secretory vesicles, particularly intense in older cells. Fixation was followed by permeabilization with Triton 0.2% for 30 min at room temperature. Cells were washed again with PBS and incubated for 30 min at room temperature with 10% normal goat serum (GIBCO, Life Technologies, MD) to mask nonspecific binding sites. Following this, chromaffin cells were incubated overnight at 37°C with rabbit anti-α_{1C} and -α_{1D} primary antibodies (1:50; Alomone Labs Ltd., Jerusalem, Israel). Chromaffin cells were then rinsed with PBS and incubated for 1 h at room temperature with a Cy3-conjugated goat anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Images were acquired using a Coolpix 990 digital camera (Nikon) adapted to the inverted Eclipse TE200 microscope equipped with 40X phase contrast objectives (Nikon Instruments SpA, Florence, Italy). Similar immunocytochemistry protocol was used for rat insulinoma β-cells.

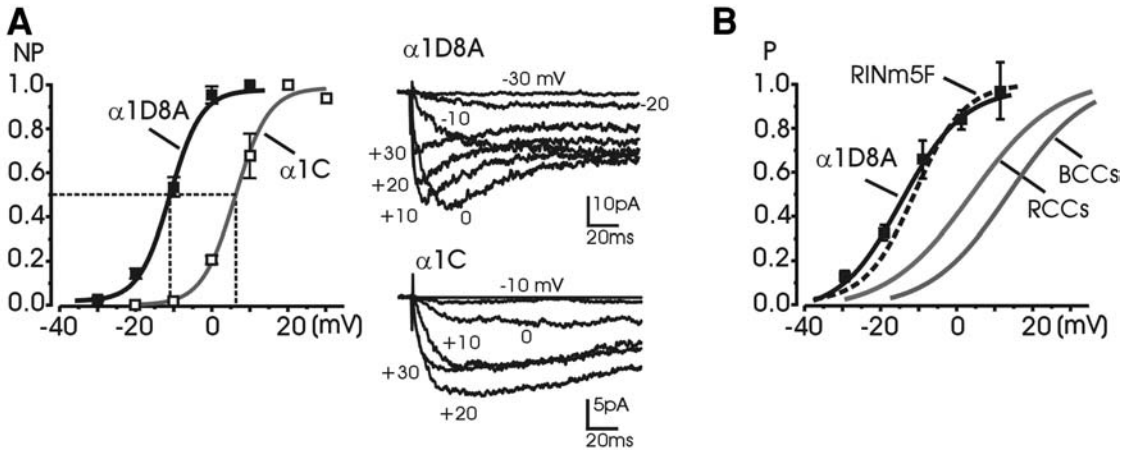

Fig. 8. Distinct voltage-dependent activation of α_{1D8A} and α_{1C} subunits transiently transfected in HEK 293 cells: a comparison to the native L-channels of RINm5F, RCCs and BCCs. **(A)** Probability of channel opening (NPo) derived from cell-attached patches containing multiple α_{1C} or α_{1D8A} channels transiently transfected in HEK 293 cells according to the procedure described in *(105).* The rabbit α_{1C} and the human pancreatic α_{1D8A} subunits were co-expressed with the rabbit $\alpha 2\delta$ and rat $\beta 3$ subunits, kindly provided by Dr. Joerg Striessnig. To the right are shown a set of Ba^{2+} currents, which are averaged over 15 traces. The normalized NPo is calculated by dividing the peak amplitude of each trace (<Ip>) by the single channel current (i) derived from measurements of single-channel currents according to the equation <Ip> = i N Po. The experimental data are fitted with a Boltzmann equation NPo = 1/[1 + exp ($V_{1/2}$–V)/k] with $V_{1/2}$ = –11 mV and k = 4.1 for α_{1D8A} and $V_{1/2}$ = +6.5 mV and k = 4.5 mV for α_{1C}. Data are means ± S.E.M. for n=5 cells. **(B)** The open probability (Po) of the single α_{1D8A} channel subunit is compared with the open probability of single native L-channels present in RINm5F cells *(95),* bovine chromaffin cells *(14),* and rat chromaffin cells (Baldelli, Carbone, Cesetti, and Hernandez-Guijo, unpublished observations). The Po vs V curve for α_{1D8A} was derived from 3 to 29 patches containing single-channel openings. The Boltzmann curves have $V_{1/2}$ = –14.2 mV (k = 6.7 mV), –10.4 (7 mV), +3.5mV (11 mV) and +13.7 mV (10.1 mV) for α_{1D8A}, RINm5F, RCCs and BCCs, respectively. Notice the close overlapping of Po(V) for α_{1D8A} and the L-channel of RINm5F cells compared to the Po(V) of the L-channels of RCCs and BBCs, which is shifted to the right. The ionic conditions for single and multiple-channel recording in HEK cells are similar to those reported in *(14).* 5 μ*M* Bay K 8644 was always present in all the recordings.

of cells predominantly expressing functional α_{1D} channels and, thus, displaying I–V characteristics with very negative activation voltages are the RINm5F cells *(95,96,106),* the adrenal glomerulosa cells *(43),* the small-cell lung carcinoma GLC8 cells *(107),* the mouse sinoatrial node myocytes *(101,102),* the amphibian hair cells *(108,109),* and the mouse cochlear inner hair cells *(110).* In 10 m*M* Ba^{2+}, these currents start activating around –60 mV, reach half maximal amplitude around –30 mV, and peak at approx –20 mW. Such negative voltage ranges of activation are observed neither in cat *(111)* nor in bovine chromaffin cells *(11),* although somewhat more negative values are observed in RCCs *(13).*

To test whether these considerations are also valid at the single-channel level, the authors ran a series of experiments comparing the activation properties of the human pancreatic α_{1D8A} subunit *(105)* and the cardiac rabbit α_{1C} subunit *(112).* Figure 8 shows the voltage-dependence of the probability of opening (NPo) of α_{1D8A} and α_{1C} subunits transiently cotransfected with the α_2-δ and β_3 subunits in HEK 293 cells. In both cases, the cell-attached patches contained multiple channels, which gave rise to macroscopic-like currents in 100 m*M* Ba^{2+} (Fig. 8A). The

selected patches contained high densities of channels (10–60 channels/μm^2) and the NPo curve shown to the left was derived at the peak of the currents. From the recordings, it is evident that α_{1D8A} starts activating at –30 mV and reaches peak values at 0 mV, whereas the α_{1C} type activates and reaches peak values at 20 mV more positive voltages. More interesting are the data of Fig. 8B in which the P_O curve derived from patches containing single α_{1D8A} channels is compared to the P_O derived for native single L-channels of RINm5F cells *(95)*, BCCs *(14)*, and RCCs recorded under similar ionic conditions. Notice the close overlapping between the P_O curve of the α_{1D8A} subunit and the L-channel of RINm5F cells in comparison to those expressed by RCCs and BCCs; the latter being the most shifted to the right. Thus, chromaffin cells appear to express L-channels with biophysical properties more similar to the α_{1C} type, whereas in the specific case of Fig. 8, RINm5F cells predominantly express the α_{1D} type.

The difference is even more striking if comparing the single-channel data of the α_{1D} type expressed in bullfrog hair cells *(109)*. The P_O curve in 65 m*M* Ba^{2+} has half maximal activation ($V_{1/2}$) at –16 mV, i.e., at about 25–30 mV more negative voltages than the L-channels of BCCs and RCCs. Mean open times are a factor 2–3 larger (in the absence of Bay K 8644) and averaged currents are non inactivating during pulses of 400 ms. Given the above findings, it is evident that the majority of the functioning L-channels in chromaffin cells are most likely associated to the α_{1C} subunit. This seems true for BCCs *(14,113)* and RCCs (Baldelli, Carbone, Cesetti, Hernández-Guijo, unpublished results) in which single-channel recordings do not indicate the presence of two clearly distinguishable L-channel classes. Whether the α_{1D} channels are overlooked in the authors' recording conditions or the channels are expressed (Figs. 6, 7) but not functioning remains to be clarified in future works. According to the present data, the most reasonable conclusion is that the two signaling pathways (G protein-mediated inhibition and cAMP/PKA-mediated potentiation) converge on the same channel type, most likely α_{1C}.

L-Channel Modulation and Exocytosis

Given that chromaffin cells express α_{1C} and α_{1D} channels, which undergo up- and down-modulation of their gating properties, the next interesting issue relates to how these modulatory mechanisms interfere with the exocytotic activity of the cells. Surprisingly enough, there are few papers on this issue, and even less on the specific role of L-channels. This is likely due to the complexity of combining simultaneous recordings of Ca^{2+} currents and exocytosis *(114,115)*. Most probably, the number will soon increase, as the methodology will improve.

The G Protein-Mediated Inhibition of Secretion and L-Channel Gating

Excitation-secretion coupling in chromaffin cells is triggered by increases of cytosolic $[Ca^{2+}]_i$ mainly associated to Ca^{2+} influx through voltage-gated Ca^{2+} channels. Since L-channels, as well as N- and P/Q-channels, are involved in the control of exocytosis, their modulation by neurotransmitters may represent an effective mechanism for regulating secretory responses. This would be particularly critical in rat, mouse, and human chromaffin cells, which express high percentages of L- over non-L-type channels *(116)*. In addition, the regulation of neurosecretion by GPCRs may occur either through the modulation of voltage-gated Ca^{2+} channels or directly on the secretory machinery (downstream of Ca^{2+} entry), thus introducing a further degree of variability to the phenomenon.

Ca^{2+}-channel inhibition by ATP and its effect on depolarization-evoked secretion has been extensively studied, in BCCs and RCCs by combining membrane capacitance measurements and whole-cell current recordings

(22,117,118). Whereas ATP is shown to inhibit the various Ca^{2+} currents by different extents (75% the L-types and ~50% the N-, P/Q-types), the neurotransmitter does not modify their efficacy to promote secretion. ATP neither alters the Ca^{2+}-dependent fusion of vesicles to the plasma membrane nor the vesicle supply to release sites, thus confirming that the ATP-induced inhibition of exocytosis is primarily associated to its action on Ca^{2+} channels *(117).* ATP reversibly inhibits Ca^{2+} currents and the related exocytotic response, suggesting a possible role for an autocrine/paracrine inhibition of Ca^{2+} channels in catecholamine release from BCCs *(118).* The action of ATP on exocytosis appears more complex in RCCs *(22).* ATP inhibits exocytosis by either depressing Ca^{2+} currents (L, N, and P/Q) or by directly acting on the secretory machinery through a Ca^{2+}-independent pathway. Both actions are mediated by PTX-sensitive $G_{i,o}$ proteins linked to P_{2Y} receptors, but the latter occurs independent of Ca^{2+} channels and accounts for most of the inhibitory effect on exocytosis induced by ATP.

The cAMP/PKA-Mediated Potentiation on Secretion and L-Channel Gating

The effects of cAMP on secretion in chromaffin cells are quite heterogenous. Some reports point to a marked increase of basal and stimulus-evoked secretion from adrenal chromaffin cells together with an increased Ca^{2+}-entry through L-channels induced by cAMP, PACAP (pituitary adenylate cyclase-activating polypeptide), and forskolin *(119–122).* To the contrary, other data support the existence of an inhibitory action of cAMP on nicotine-induced release and Ca^{2+} currents in BCCs *(123–125).* In some works, L-channels and membrane voltage are shown to play an exclusive role in increasing the stimulus-induced secretion by cAMP *(126),* while in others the role of these components appears more limited or unnecessary *(120,127).*

Among this complex pattern of responses, the contribution of Ca^{2+}-channels and their modulation by cAMP in the control of exocytosis has recently been investigated in RCCs *(21).* The cAMP permeant analog pCPT–cAMP is found to potentiate both the L-currents and the depolarization-evoked secretion, but the current increase accounts for only 20% of the total secretory response. cAMP doubles the size of the readily-releasable pool (RRP) of vesicles by almost doubling the mean size of unitary exocytic events (from 1.1 to 2.1 fF; *see also* ref. *122*), without affecting the probability of release. cAMP potentiates the secretion independent of the Ca^{2+} channel type activated and the same effects are induced by β_1-AR stimulation through a PKA-mediated pathway. All these data support the hypothesis of a positive feedback signaling, which is activated by the cAMP/PKA pathway and is likely to control the fast release of catecholamines during sustained cell activity.

Conclusions

Given the critical role of L-type Ca^{2+} channels in controlling cell excitability and neurotransmitter release, it is not surprising that this class of channels is extensively regulated by a number of signaling pathways. The work of the last five years has shown that neuroendocrine L-channels undergo a marked autocrine modulation induced by the material released during cell activity, causing either inhibition or potentiation of the Ca^{2+} current controlling exocytosis. The most original part of this action is the existence of a direct GPCRs-mediated inhibition of L-channels, which co-exists with the classical up-regulation mediated by the cAMP/PKA-signaling cascade. In chromaffin cells, this combination of opposing modulations of L-channels has the advantage of allowing the increase of intracellular cAMP by means of β-ARs stimulation, with consequent increased exocytosis *(21)* and recruitment of newly synthesized T-type and Na^+ TTX-resistant channels *(76),* without significantly altering the intracellular Ca^{2+} levels (*see* Fig. 9). The overall effect of L-channels auto-regulation by released neurotransmitters

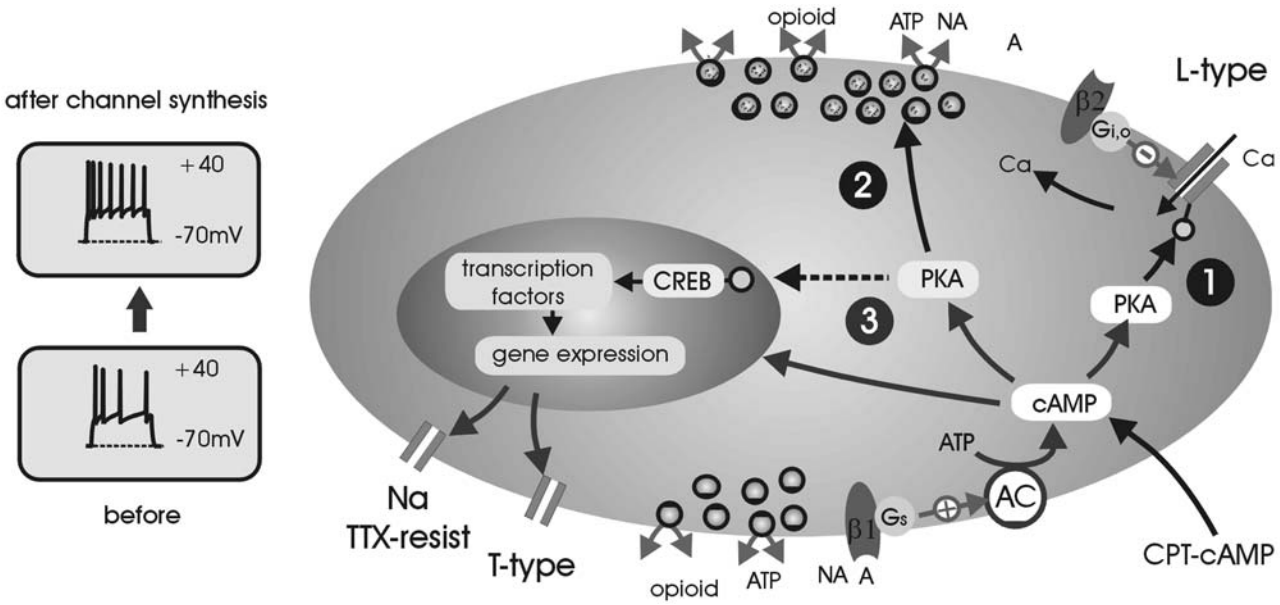

Fig. 9. Schematic drawing of the signaling pathways associated with β_1-AR and β_2-ARs stimulation on RCCs. β_2-ARs are negatively coupled to the L-channel through a PTX-sensitive $G_{i,o}$ protein via a direct membrane-delimited pathway. β_1-AR causes the enhancement of cAMP levels, which activate three distinct signaling pathways and remote effectors: 1) a PKA-mediated phosphorylation of L-channel α_1 subunits leading to an increased gating activity occurring within few minutes *(8),* 2) an increased PKA-dependent potentiation of exocytosis occurring downstream and independently of the Ca^{2+} current increases *(21)* and, 3) a long-term (2–3 d) cAMP-dependent recruitment of newly synthesized low-threshold T-type Ca^{2+} channels and Na^+ TTX-resistant channels *(76)* which lowers the threshold of action potential firings and cell excitability.

(adrenaline, noradrenaline, ATP, and opioids) is therefore an enhancement of cell activity and neurotransmitter release with little changes to $[Ca^{2+}]i$, which may result deleterious for cell survival during maximal sympathetic stimulation and sustained catecholamine release (fight-or-flight response). Thus, as recently suggested *(8),* the complexity of L, N, and P/Q-channel modulation may just work in concert for protecting chromaffin cells from Ca^{2+} elevations and cell apoptosis while preserving maximal functionality.

Most neuroendocrine cells possess α_{1C} and α_{1D} L-type channels whose degree of expression changes from cell to cell and with time, depending on various factors. It is not clear yet the exact role of these two classes of channels, and due to the difficulty of pharmacologically separating the two channel types, the solution of this issue will require new molecular biology approaches combined with more detailed biophysical single-channel studies.

Acknowledgments

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