

Expression and Role of T-type Calcium Channels during Neuroendocrine Differentiation

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Abstract

Neuroendocrine cells release their secretion into the extracellular environment through calcium-dependent signalling pathways. These cells share common morphological and molecular features such as the expression of specific biomarkers, neurite outgrowth and dense core secretory granules. In order to elucidate the signalling pathways leading from undifferentiated to differentiated neuroendocrine cells, the role of voltage-dependent calcium channels, central actors in the excitation-secretion coupling, has been comprehensively investigated. T-type calcium channels appear to belong to the most important ion channel family involved in the neuroendocrine differentiation process. They could also participate in the development of neuroendocrine tumours.

Keywords Calcium channels; Neuroectoderm; Neuroendocrine

Introduction

Neuroendocrine (NE) cells produce and release (neuro) hormones, neuropeptides or monoamines in the extracellular milieu using a regulated pathway in response to a specific stimulus. The NE system includes specific organs, where cells are organized into secreting tissues (adrenals or pituitary for instance) and a diffuse system, where endocrine cells are scattered in an apparently disorganized pattern among non-secretory cells. The observation of dispersed NE cells throughout the body derives from the seminal works of Heidenhain, Kulchitsky, Masson and Hamperl in the late 19th century and the early 20th century. Their studies demonstrated, from the ability of cells to be stained by silver salts (argent affinity or argyrophily), the existence of dispersed hormone-secreting cells in non-endocrine tissues (for a review see [1]). Using histological techniques, Feyrter and Froelich [2,3] identified the existence of many clear cells (Hellen Zellen) throughout the body and noticeably in the gut, leading to the concept of DNES. Miscellaneous sites were also identified in the prostate, where they are dispersed in the normal epithelium, representing only about 0.5 to 2 % of the total cell population [4]. Anton Pearse then defined NE cells as cells sharing the common ability to uptake and decarboxylate amine precursors, to convert these precursors into biogenic amines and neurotransmitters. From this property, Pearse [5] coined the term, APUD system (Amine Precursor Uptake and Decarboxylation). NE cells were all initially thought to originate only from neuroectoderm (neural crest) and were thus also termed para neurons [6]. However, subsequent studies showed that some of them did not derive from the neural crest. These include pancreatic endocrine cells [7] or gastrointestinal enterochromaffin cells [8,9], which originate from endodermal tissue.

NE cells are usually characterized by various functional, morphological and molecular features (for a review see [10]). A NE cell is characterized in electron microscopy by the presence of electrodense secretory granules containing peptidergic and aminergic

neurohormones. NE cells possess the capability to rapidly release their neurohormones or neurotransmitters in response to specific stimuli, such as other hormones. In this context, the acquisition of cell excitability, characterized by action potentials and voltage-dependent ion channels, is an important step towards the acquisition of a regulated secretory pathway. Morphologically, the acquisition of a NE phenotype is usually accompanied by the lengthening of neuritic extensions. NE cells therefore share many features with neurons, but do not have specialized nerve terminals or axons. From a molecular point of view, NE cells express specific proteins that may also be expressed by neurons or even by non-neuronal cells. Among these NE markers are the members of the granin family, chromogranin A and B (CgA and CgB), secretogranin II, III and VII. These acidic soluble proteins are stored with neuropeptides and neurotransmitters in dense-core vesicles. Other markers of NE differentiation are the enzymes Prohormone Convertases PC1/3 and PC2, which process various protein precursors, including granins, into functional hormones [11]. Like granins and neuropeptides, Prohormone Convertases are stored in dense-core vesicles where they exert their proteolytic activity. These enzymes have therefore been considered as good markers of the NE phenotype or differentiation [12]. These common markers, along with others (neuron-specific enolase (NSE), synaptophysin, S100), are used in pathology to identify NE tumours. Specific features of neuronal or NE differentiation occurring during development (neuritic outgrowth or excitability for instance) are associated with changes in the expression of various ion channels [13]. For instance, transient calcium variations due to ion channel activity control various aspects of neuronal development. Indeed, rapid calcium spikes (relying on voltage-dependent ion channels and intracellular ryanodine receptor channels) and slow calcium waves (involving calcium entry at resting membrane potential amplified by intracellular calcium release) are involved respectively in the expression of neurotransmitter and axon outgrowth in the developing neurons [14]. A variety of ion channels, including voltage-dependent sodium [15] or calcium channels [16], voltage-dependent potassium channels [17], mechanosensitive channels and TRP channels [18-20]

have been described as actors of neurite formation. In this context, as we will see later in this review, T-type calcium channels play an important part in neurite lengthening occurring during neuroendocrine or neuronal differentiation.

Cell Models for NE Differentiation

NE differentiation leads to cell phenotypes sharing characteristic features of both developing neurons (neurite extension, neuronal markers, and synaptic-like vesicles) and endocrine cells (secretory granules, neurohormones). Therefore, NE differentiation has been investigated using various cell models.

One of the most studied cell models for NE differentiation is the PC12 cell line, which is a valuable model for neurosecretion studies [21]. Like normal chromaffin cells, PC12 cells synthesize and re-lease monoamines such as dopamine and noradrenaline, in a calcium-regulated manner. Upon stimulation for several days with Nerve Growth Factor (NGF), PC12 cells differentiate into neuronal-like cells [22]. Morphological differentiation characterized by neuritic extension, is associated with reduced or halted proliferation and with increased tyrosine hydroxylase activity. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) also leads to NE differentiation characterized by similar features (for a review see [23]). Furthermore, NE differentiation can be induced in these cells by dexamethasone, a potent synthetic glucocorticoid, which induces the overexpression of tyrosine hydroxylase, leading to a chromaffin phenotype, potentiating calcium-dependent exocytosis through an increase in vesicle content and calcium channel coupling to release sites [24]. The LNCaP prostate cancer cell line is also a model of choice for studying NE differentiation. The first study showing LNCaP cell differentiation towards a NE phenotype demonstrated that permanent analogues of cyclic AMP (cAMP) could promote neurite extension and the expression of NE markers, CgA, NSE and S100 protein [25]. As in PC12 cells, NE differentiation was associated in LNCaP cells with an enhanced survival and a lower proliferation rate. NE differentiation was later shown to be induced in LNCaP cells by the depletion of steroid [26,27], or by the addition of interleukins (IL) 1, 2 or 6 [28,29] to the culture medium. Various neuropeptides, such as Vasoactive Intestinal Peptide (VIP) [30-32], PACAP [33], melatonin [34] or adrenomedullin [35], also promote NE differentiation of LNCaP cells.

LNCaP and PC12 cells thus share many common features that make them valuable and easy-to-use cell models for studying NE differentiation. Nonetheless, they are each characterized by specific differentiation patterns. For example, NGF, which is a good inducer of NE differentiation in PC12 cells, does not promote a neuronal phenotype in prostate cancer cells [36]. In addition to these two cell lines, a number of other cell models have been used to study an

important aspect of neuronal differentiation, i.e. neuritogenesis, notably neuroblastoma cell lines, retinoblastoma cell lines, embryonic stem cells or neurons [37-39].

Signalling pathways and role of calcium

This topic falls beyond the purpose of this review. Therefore, only the main signalling pathways common to LNCaP and PC12 cells will be rapidly described here. Some of the pathways leading to NE differentiation are depicted in Figure 1. In both cell lines, the activation of a cAMP-dependent pathway is clearly shown to induce a neuronal phenotype. In LNCaP cells, the induction of a neuronal phenotype by stimuli raising the cytosolic cAMP concentration clearly depends on cAMP-dependent protein kinase (PKA) [40]. cAMP-dependent pathway was later shown to involve the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway [41], which then phosphorylates downstream targets like the cAMP responsive element binding protein (CREB) [33]. The activation of the ERK1/2 pathway by heparin-binding epidermal growth factor-like growth factor (HB-EGF) also induces NE differentiation of LNCaP cells [42]. In PC12 cells, it has been shown that specific stimulation of EPAC (exchange proteins activated by cAMP) promotes neuritogenesis, whereas specific PKA activation leads to the ERK1/2 pathway and cell proliferation [43]. On the other hand, PACAP stimulates neurite outgrowth via ERK activation [44] and the NF- κ B pathway [45]. In addition, the activation by androgen removal of the PI3K-Akt-mTOR pathway may be essential to induce NE differentiation in LNCaP cells [46], as is the case for neuritogenesis-induced NGF in PC12 cells [47].

In addition to the signalling pathways described above, calcium ion has been proposed as a key messenger involved in NE differentiation. In LNCaP cells, it has been shown that VIP, a neuropeptide inducing the expression of NE markers, such as NSE and CgA [30,32], also rapidly increases cytosolic calcium levels, c-Fos proto-oncogene and VEGF mRNA expression. This is associated with VEGF secretion and neurite lengthening. All these effects were shown to be antagonized by chelating intracellular calcium using BAPTA-AM [31], which therefore clearly demonstrates that NE differentiation is a calcium-dependent process. It has also been shown in the same cells, that NE differentiation, induced by either steroid depletion or cytosolic cAMP elevation, is associated with a profound alteration of calcium homeostasis [48]. Indeed, NE differentiation is associated with reduced endoplasmic reticulum calcium store content, which is attributed to decreased calreticulin (a calcium/storage binding protein) expression and SERCA2b-Calcium ATPase [48]. In addition, this has been correlated with smaller capacitative calcium entry [48-51] and to an increased resting cytosolic calcium concentration [52,53].

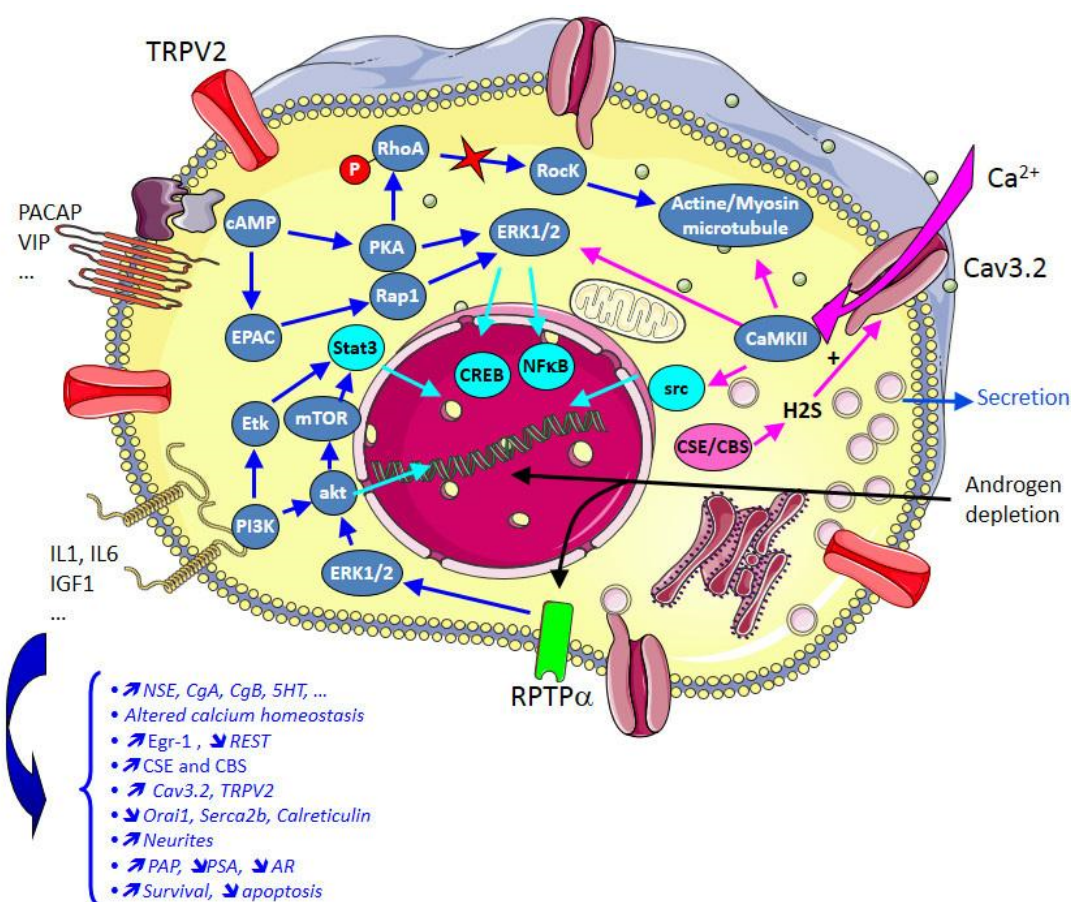


Figure 1: Overview of the transduction pathways leading to NE differentiation in LNCaP cells. NE differentiation can be induced in LNCaP cells by peptides activating G-protein Coupled Receptors (GPCR) (PACAP, VIP, bombesin...), tyrosine kinase receptors (NGF, IGF1, IL1, IL6...) or androgen depletion through increased expression of Receptor Protein Tyrosine Phosphatase Alpha (RPTPα) [119]. PKA/ERK/CREB [40], PI3K/Akt/mTOR [46], PI3K/Etk/Stat3 pathways activate NE. Differentiation characterized by the features described below (for a review see [120]). Phosphorylation of RhoA by PKA leads to its inactivation, to Rho kinase (Rock) inhibition and to neurite extension [121]. Egr-1 is overexpressed during NE differentiation which promotes the ensuing over expression of Cav3.2 channels. Calcium entry through Cav3.2 channels may increase cytosolic calcium concentration, thus leading to activation of CaM kinase II and further activation of Src, leading to gene expression. CaM kinase II stimulation may also promote the cytoskeleton reorganization necessary for morphological differentiation. As shown, other channels like TRPV2 may be involved in NE differentiation [53].

In PC12 cells, NE differentiation is also associated with cytosolic calcium increases upon the application of the stimulus. For instance, PACAP promotes an elevation of the intracellular calcium concentration, which is an essential step towards the acquisition of the NE phenotype. Selenoprotein T (SelT), a selenocysteine, is an essential player in this calcium-dependent differentiation. Its expression is enhanced by PACAP [54] and this requires calcium entry. In addition, SelT is involved in calcium homeostasis since its overexpression increases the intracellular calcium concentration, whereas its knockdown reduces PACAP-induced calcium signals [54].

Expression of voltage-dependent calcium channels

Since NE cells are characterized by electrical excitability, voltage-dependent calcium channels have been considered as putative actors of the NE differentiation process. Voltage-dependent calcium channels

are classified, based on their biophysical and pharmacological properties, as Low-Voltage Activated calcium channels (LVA or T-type calcium channels) and High-Voltage Activated calcium channels (HVA or L, N, P/Q, R-type calcium channels). On a molecular basis, ten different $\alpha 1$ pore subunits have been identified that present voltage-dependent calcium channel properties [55]: Cav1.1, Cav1.2, Cav1.3, Cav1.4 (L-type channels), Cav2.1 (P/Q-channels), Cav2.2 (N-type), Cav2.3 (R-type), and Cav3.1, Cav3.2, Cav3.3 (T-type channels). Regarding the expression levels of voltage-dependent calcium channels, their involvement in neuronal or NE differentiation has been investigated in various cell models displaying the initial steps of differentiation. LVA calcium channels have thus been shown, along with HVA calcium channels, to be differentially regulated during NE or neuronal differentiation. It has been shown in the literature that in many neuronal models, T-type calcium channels are expressed in the

early stages of development and may participate in the differentiation process [56,57]. The involvement of calcium channels, notably T-type calcium channels, in neuronal development has been reviewed thoroughly elsewhere [58].

In the neuroblastoma X glioma hybrid NG108-15 cell line, it was shown from electrophysiological experiments and pharmacological evidence [59] that only T-type calcium channels are expressed in the undifferentiated state. Moreover, neuronal differentiation induced by prostaglandin and isobutyl-methylxanthine (IBMX) is accompanied by an overexpression of T-type calcium channels and the neo-expression of other voltage-dependent calcium channels (L, N, Q-type channels). These results led to the hypothesis that T-type calcium channels may be some of the channels involved in triggering neuronal differentiation (see below). SN56 cells, another hybrid cell line resulting from the fusion of neuroblastoma cells and septal neurons, express voltage-dependent calcium channels upon neuronal differentiation. In their undifferentiated state, SN56 cells mostly express T-type calcium channels [38]. Based on their kinetics and their pharmacological sensitivity to nickel, the authors suggested that T-type calcium currents were carried by Cav3.2 pore subunits. Upon differentiation induced by serum deprivation and cAMP, an increase in T-type calcium currents was observed within 2 to 4 days, together with a *de novo* expression of L, N, P/Q and R-type HVA calcium channels [38]. As in NG108-15 cells, it is therefore hypothesized that differentiation is dependent on T-type calcium channel activity in SN56 cells. In another human neuroblastoma cell line B2 (2)-M17, neuronal differentiation (evidenced by an increased expression of NSE, $\alpha 7$ -ACh nicotinic receptor and SNAP-25) induced by retinoic acid, leads to increased voltage-dependent calcium entry upon KCl depolarization [60]. Based on pharmacological demonstrations, this was mainly attributed to N, P/Q channels together with a slight participation of T-type calcium channels.

In the human retinoblastoma Y-79 cell line, where both Cav3.1 and Cav3.2 are expressed in undifferentiated cells, there is an important reduction in mRNA levels for both channels, along with a diminution of T-type calcium current in cells undergoing neuronal differentiation [39]. Down-regulation of Cav3.1 T-type channel activity and expression was further demonstrated to be due to a reduced activity of the promoters controlling its transcriptional expression [61]. A more recent study analysing modifications in alternative splicing showed that there is a considerable change in Cav3.1 splice variants occurring during neuronal differentiation [62]. Nevertheless, knocking-down the expression of Cav3.1 failed to alter differentiation kinetics or neurite formation. These results led the authors to suggest that Cav3.1 and Cav3.2 channels are not involved in the differentiation process itself, but that the alterations in splice variant specific expression may be relevant for establishing a mature differentiated state [39].

In embryonic stem (ES) cells, neuronal differentiation promotes electrical excitability and is associated with maturation of voltage-dependent calcium channels [63]. This differentiation can be induced by various neuropeptides, including VIP or PACAP. Both peptides stimulate neurite outgrowth and NSE overexpression after 4-8 days treatment [64]. This is associated with an enhancement of T-type calcium currents [65] correlated with a stronger expression of Cav3.3 protein as observed in western-blot experiments.

It has been shown in PC12 cells that NE differentiation is accompanied by increased expression of both LVA [66] and HVA calcium channels [67,68]. Chronic application of various agents that induce NE differentiation, such as NGF or dexamethasone, leads to an

increase in both the proportion of cells expressing LVA calcium currents and the average LVA calcium current density [66]. NE differentiation induced by PACAP or NGF is associated with increased density of voltage-dependent calcium currents, both of the LVA and the HVA types [69].

In the NE pituitary lactosomatotrope GH3 cell line, further differentiation towards a lactotrope phenotype may be induced by treatment with NGF for several days. While a short treatment with NGF only up-regulates HVA calcium channels, a sustained NGF action (one to 5 days), occurring through a p75 receptor, leads to up-regulated prolactin mRNA and protein levels, as well as to a delayed increase in T-type calcium currents [70]. These T-type calcium currents are possibly carried by Cav3.1 and/or Cav3.3 channels since they are the only LVA subunits expressed in GH3 cells [71].

In the LNCaP prostate cancer cell line, previously shown to differentiate following cAMP stimuli [25], it was demonstrated those 3 to 4 days after the onset of NE differentiation, there was an increase in the magnitude of voltage-dependent calcium currents. This calcium current displayed all the characteristics of T-type calcium channels [52]. Indeed, it was characterized by a fast and voltage-dependent inactivation, a slow deactivation as well as being inhibited by low concentrations of NiCl_2 , mibefradil, flunarizine and kurtoxin. The increase in T-type calcium current took longer to occur than the lengthening of neuritic extension which developed within a few hours of the early steps of differentiation. It was demonstrated by quantitative RT-PCR that the enhancement of T-type calcium currents depends on an overexpression of Cav3.2 channels. A particular feature of LNCaP cells is that Cav3.2 calcium channels are the only voltage-dependent calcium channels expressed in both undifferentiated and differentiated conditions. Cav3.2 overexpression was associated with an increase in resting cytosolic calcium concentration, antagonized by T-type calcium channel inhibitors [52]. It was shown that T-type calcium channels, due to their combined inactivation and activation properties, participate in basal calcium entry at a resting membrane potential of -40 mV in LNCaP prostate cells [72]. The overexpression of Cav3.2 channels was shown to be mediated by a transcriptional mechanism in response to the activation of adenylyl/PKA-Egr-1 pathway [52,73], to androgen depletion [52] or to sodium butyrate exposition [74]. On the opposite, it was shown that interleukin 6 (IL6), which also promotes NE differentiation, increases Cav3.2 channels through a post-transcriptional mechanism [75].

Role of T-type calcium channels in NE differentiation

NE phenotype is characterized by neurite formation and calcium-dependent secretion. Many studies have thus investigated whether T-type calcium channels may be involved in these features.

Role of calcium in neurite formation and extension

There has been some debate concerning the positive or adverse effects of intracellular calcium and voltage-dependent calcium channels on neuritic extension. While some published studies show no role for voltage-dependent calcium channels in neurite extension induced by NGF [76], most publications report a stimulation of neurite outgrowth by increased cytosolic calcium levels due to calcium entry through several channels. Indeed, in many cell models, calcium is necessary for neurite out-growth. For instance, it has recently been shown in PC12 cells that PACAP is able to induce neuritogenesis through a NF- κ B pathway. The authors demonstrated that PACAP-

induced NE differentiation occurs through the activation of a calcium-dependent ERK1/2 MAP kinase pathway and the recruitment of cRel and p52 subunits of NF- κ B, leading to neuritogenesis and cell survival [45]. In that study, neuritogenesis and cRel recruitment are both inhibited by non-specific inhibitors of plasma membrane calcium channels and intracellular calcium channels responsible for endoplasmic reticulum calcium release. In PC12 cells, calcium homeostasis perturbation, for example induced by knocking-down the expression of plasma membrane Ca^{2+} ATPase (PMCA 2 and 3), leads to alterations in the process of morphological differentiation characterized by neuritic extension [77].

Role of T-type calcium channels in neurite formation and extension

T-type calcium channels have thus been investigated as putative key players of the differentiation process since, as described above, they are frequently expressed in either the early or late stages of NE/neuronal differentiation. The role of T-type calcium channels in neurite formation has been investigated in embryonic neural progenitor cells [78]. From the inhibition of calcium signals induced mainly by T-type channels inhibitors (mibefradil, NNC-550396), it was concluded in this study that T-type calcium channels are expressed during the early stages of neuronal differentiation (one day).

However, during the differentiation process, there is an increase in the density of HVA channels compared to LVA channels, such that at differentiation day 20, calcium signals are totally inhibited by nifedipine. The authors showed that inhibitors of LVA channels significantly decrease the number of active migrating neuron-like cells and neurite extensions, thereby demonstrating their involvement in neuritogenesis. Early reports on neuroblastoma cells demonstrated that the expression of T-type currents always precedes neurite extension, thus suggesting that calcium entry through T-type calcium channels may participate in morphological neuronal differentiation [79]. In the NG108-15 cell line, it was shown that cAMP-induced neuronal differentiation, characterized by neurite formation and an overexpression of L-type calcium channels, is dependent on T-type calcium channels activity. Indeed, the inhibition of T-type calcium channels using NiCl_2 or the down-regulation of the Cav3.2 pore subunit, led to a decrease in the number of cells with neurite, but did not change the average neurite length [80]. In addition, Cav3.2 T-type calcium channels were necessary players in HVA calcium channel expression during neuronal differentiation. This led the authors to propose that Cav3.2 T-type calcium channels are an early actor in the neuronal differentiation process.

In prostate cancer LNCaP cells, neurite formation induced by cAMP is faster (occurring in few hours) than Cav3.2 overexpression (occurring in few days), which suggests that Cav3.2 overexpression may only be a consequence of the differentiation process. However, there is significant expression of Cav3.2 channels and T-type calcium current, in the undifferentiated state which could be responsible for the morphological differentiation process. We have indeed shown that neurite elongation during NE differentiation is dependent on Cav3.2 calcium channels since their inhibition by low NiCl_2 concentrations reduces the average neurite length without hampering neurite formation. Therefore, if neurite formation during cAMP-induced NE differentiation is not dependent on Cav3.2 channels, their lengthening is modulated by T-type calcium channel activity [52]. In a recent study, it has been similarly shown that IL6 or Na-Butyrate, a histone deacetylase inhibitor, induce NE differentiation and that neurite

branching and lengthening are partially dependent on Cav3.2 activity [74].

Role of T-type calcium channels in secretion

The central role of cytosolic calcium in the regulated pathway of secretion (and therefore in hormones, neurohormones and neurotransmitter release) has been demonstrated in many endocrine, NE or neuronal cells.

The role of HVA channels in regulated exocytosis has been known for a long time, but attention has been paid more recently to LVA channels and their role in calcium-dependent secretion or neurotransmission has now been documented [81]. Anterior NE pituitary cells express both LVA and HVA calcium channels [82] and although HVA channels regulate hormone secretion, pharmacological experiments on perfused rat pituitaries have demonstrated that T-type calcium channels also participate in the secretion of hypophyseal hormones like Thyrotropin, in response to hypothalamic neuropeptides [83]. In addition, in NE pituitary cells, the expression of Cav3.1 is raised, whereas the expression of Cav3.2 and Cav3.3 is reduced in mice treated with 17β -estradiol. This regulation of T-type calcium channel mRNA expression could be an important mechanism involved in the 17β -estradiol regulation of pituitary secretion [84]. A convincing link has been demonstrated for L-type calcium channels and exocytosis in many NE cell models. However, it has been shown in various NE cells, including rat pituitary melanotropes, that T-type calcium channels are as efficient as L-type channels in promoting exocytosis. In these cells, calcium ions “couple with equal strength to exocytosis regardless of the channel type involved” [85].

In prostate NE LNCaP cells, we have shown that membrane depolarization, due to a stimulation of T-type calcium current, induces calcium-dependent exocytosis, as measured with FM1-43 dye. In addition, we showed that prostatic acid phosphatase (PAP) secretion is calcium-dependent in prostate cells and that this secretion is potentiated by NE differentiation. Furthermore, PAP secretion is reduced by low concentrations of NiCl_2 , flunarizine or kurtoxin and by siRNAs targeting Cav3.2 channels. Cav3.2 expression and activity are therefore responsible for PAP secretion in NE prostate cancer cells [86]. In addition, it has been demonstrated that Cav3.2 channels are activated by hydrogen sulfide (H_2S) which is produced by an overexpression of cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS) during NE differentiation of LNCaP cells [73]. This in turn leads to increased secretory function of NE cells.

In adult chromaffin cells, the expression of LVA channels is weak and HVA channels control to a large extent voltage-dependent calcium influx and thereby catecholamine secretion [87]. However, the expression of LVA channels is stronger in embryonic chromaffin cells [88] than in adult cells. It therefore seems that during development, chromaffin cells lose the capacity to express LVA channels, which are progressively replaced by HVA channels. In adult cells, the Cav3.2 isoform is weakly expressed but is up-regulated either by a treatment that increases cytosolic cAMP concentration [89] or by chronic hypoxia [90]. This cAMP effect was demonstrated to be mediated by a PKA-independent signalling pathway through the cAMP-receptor protein Epac ((cAMP-guanine nucleotide exchange factor) cAMP-GEF) [89]. In addition, newly-formed Cav3.2 channels are as efficient as HVA channels in promoting low-threshold exocytosis and the release of catecholamine [90-92].

In mouse chromaffin cells *in situ*, short-term exposure to PACAP stimulates T-type calcium channel activity through a PKC-dependent recruitment of Cav3.2 channels to the plasma membrane [93]. Cav3.2 channels may therefore be responsible for catecholamine secretion in acute stress situations. It has thus been widely shown that LVA calcium channels control secretion and neurotransmission. This has been discussed in a recent review [94]. However, the mechanisms involved in that function have as yet remained unclear. To promote a fast release of neuropeptides or neurotransmitters, HVA channels, for their part, are localized in close vicinity of the vesicle-release machinery. It has been demonstrated that this co-localization is achieved using a specific interaction between HVA channels (Cav2.1 and Cav2.2) and SNARE proteins (syntaxin-1, SNAP-25 and synaptotagmin-1), through an interaction site called a synprint. It has recently been shown, that although T-type calcium channels lack this synprint interaction site, they closely associate with syntaxin-1A in central neurons [95]. This association between syntaxin-1A and Cav3.2 was also demonstrated in the same study to be essential for low-threshold exocytosis in chromaffin cells.

Putative signalling mechanisms involved in T-type calcium channels mediated-NE differentiation.

The downstream events after calcium entry through T-type calcium channels in the context of NE differentiation have been rarely studied. Since T-type calcium channels have been shown to be involved in secretion, it has been investigated whether their role in the induction of a NE phenotype could be related to the secretion of growth factors. As shown by one study [96], neuritogenesis could be induced in NG108-15 cells using the conditioned media from Cav3.2 expressing NG108-15 cells. When the expression of Cav3.2 channels was knocked-down with siRNAs, conditioned media from these siRNA-treated cells did not induce neurite outgrowth in NG108-15 cells, thus showing the involvement of T-type channels in a paracrine loop promoting neurite formation. However, the nature of the factors secreted by NG108-15 cells in response to T-type channels activity has not been elucidated.

In addition, T-type calcium channels may promote NE or neuronal differentiation through a stimulation of gene expression. It has been shown in NG108-15 cells that T-type calcium channels are responsible for the induction by hydrogen sulfide (NaHS) of a neuronal phenotype, characterized by the overexpression of L-type calcium channels and neuritogenesis [97]. This was essentially demonstrated using T-type calcium channel inhibitors, such as mibefradil or zinc chloride in a range of concentrations more specifically inhibiting Cav3.2 channels. In the same cell line, it was later demonstrated that NaHS promotes neuritic extension through a phosphorylation of Src kinase [98]. Indeed, PP2, a specific inhibitor of Src, antagonized the development of neurites induced by NaHS, while only reducing the ones activated by permanent cAMP. In addition, Src phosphorylation by NaHS was significantly reduced by inhibitors of T-type calcium channels, mibefradil or ascorbate, showing that T-type calcium channels are upstream of Src in the pathway leading to a neuronal phenotype. It was shown in this study that Src phosphorylation is independent of CaMKinase II. Another study demonstrated that depolarization-induced calcium entry promotes NE differentiation of PC12 cells. This NE differentiation characterized by neurite formation, GAP-43 and synapsin I overexpression, requires the activation of phospholipase D2–ErK–CREB pathway and Src phosphorylation by CaMKinase II [99]. Such a pathway is similar to the one demonstrated in hippocampal

neurons, where NCAM (neural cell adhesion molecules) induced neurite formation is dependent on the T-type calcium channel [100]. In these cells, homophilic NCAM interaction, which promotes neurite formation, leads to an increase in cytosolic calcium via T-type calcium channels, together with L-type and non-selective cation channels, and to the activation of a Src-dependent pathway, both events being necessary for the induction of differentiation.

NE differentiation in cancer progression- Perspectives on the general involvement of T-type calcium channels

Cancer development is characterized by a panel of hallmarks (including enhanced and autonomous proliferation, cell death resistance, immortality, angiogenesis, invasion and metastasis) in which ion channels mutations or alterations have been shown to be involved [101-105].

NE tumours may arise from the proliferation of NE cells. These tumours develop in almost all tissues, including those where NE cells may not be present [1]. It must, however, be noted that the digestive system is the most frequent site of development of NE tumours (66%) followed by the respiratory tract (31%) [106]. NET, although slow-growing, are often diagnosed after the tumour has metastasized and thus may entail a poor prognosis. Some tissues or organs may also be the site for the development of carcinoma that displays NE cell foci, which has led to the notion of cancers with NE differentiation. This is the case for prostate cancer, which is frequently characterized by NE differentiation [107,108]. In prostate cancer, NE differentiation is usually correlated with a poor prognosis [109], probably due to the fact that NE cells are androgen-insensitive [110] and may thus participate in the androgen-independent growth of prostate cancer. In addition, these cells are resistant to apoptosis [48] and secrete numerous factors that may increase cell proliferation in their vicinity. In some forms of prostate carcinoma, NE cells are the main component of the tumours. These small-cell prostate carcinomas (SCPCa) are very aggressive forms of prostate cancer, where NE cells exhibit tumorigenic and highly proliferative activities [111,112]. As shown recently, SCPCa cells may arise from NE carcinoma cells from a p53 inactivating mutation, a mutation conferring proliferative properties [113].

As reported in a number of cancers (for a review see [114], T-type calcium channels may participate in tumour growth. Their role in cell proliferation may be due to the fact that enhanced expression of T-type calcium channels (Cav3.1, Cav3.2 or Cav3.3) leads to increased secretion of tumorigenic factors by NE cells. As we have previously shown, PAP synthesis and release are enhanced by Cav3.2 channels in LNCaP cells [86]. In addition, the secreted form of PAP may play an important role in the development of prostate tumour metastasis. Indeed, PAP expression is increased in prostate bone metastasis and PAP may participate in the osteoblastic phase of the metastasis development [115]. Moreover, we have shown that the expression of Cav3.2 channels is correlated with the expression of serotonin [86], a neurotransmitter stimulating the proliferation and migration of prostate cancer cell lines [116]. Furthermore, we have shown that Cav3.2 expression correlates with CgA expression [86]. The circulating level of CgA, an NE marker, is frequently increased in the advanced stages of the diseases. It has been demonstrated in prostate cancer cell lines that CgA accelerates cell proliferation and reduces cell apoptosis, thereby suggesting a role for CgA in prostate cancer development [117]. Altogether, this suggests that Cav3.2 channels in NE prostate cancer cells may promote calcium entry, which in turn induces the secretion of mitogenic or tumorigenic factors. In addition, we have

observed that Cav3.2 channels in prostate cancer LNCaP cells, favour cell growth, even in NE cells, since their overexpression accelerates cell proliferation. In contrast, down-regulation of Cav3.2, or its blockade with various inhibitors, slows cell proliferation [118]. This is in agreement with many articles showing the participation of T-type calcium channels in various aspects of cell growth, including cell proliferation or cell apoptosis (for a review see [114] or [58]). Since NE cells of the prostate may proliferate rapidly in some forms of prostate carcinomas (SCPCa), it will be of great interest to assess whether Cav3.2 channels, involved in both proliferation and NE differentiation, in combination with a p53 inactivation, are indeed involved in the development of SCPCa.

Concluding remarks

LVA calcium channels have frequently been shown to be overexpressed during NE differentiation, as well as being involved in the development of a NE phenotype characterized by morphological (neurite extension) and molecular (NSE or CgA markers) features. Moreover, T-type calcium channels, along with other calcium channels, participate in the secretion of paracrine or endocrine factors, which in turn may promote the differentiation or the proliferation of target cells. In the context of NE tumours, most of the data available rely on cell line studies. It is of particular importance in the near future to carry out major studies on tissue arrays, in order to correlate the stage of the disease with the expression of the different T-type calcium channel isoforms.

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