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Ca^{2+} Channel Toolkit in Neuroendocrine Tumors

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Abstract
Neuroendocrine tumors (NET) constitute a heterogeneous group of malignancies with various clinical presentations and growth rates but a common origin in neuroendocrine cells located all over the body. NET are a relatively low-frequency disease mostly represented by gastroenteropancreatic (GEP) and bronchopulmonary tumors (pNET); on the other hand, an increasing frequency and prevalence have been associated with NET. Despite great efforts in recent years, the management of NET is still a critical unmet need due to the lack of knowledge of the biology of the disease, the lack of adequate biomarkers, late presentation, the relative insensitivity of imaging modalities, and a paucity of predictably effective treatment options. In this context Ca^{2+} signals, being pivotal molecular devices in sensing and integrating signals from the microenvironment, are emerging to be particularly relevant in cancer, where they mediate interactions between tumor cells and the tumor microenvironment to drive different aspects of neoplastic progression (e.g., cell proliferation and survival, cell invasiveness, and proangiogenetic programs). Indeed, ion channels represent good potential pharmacological targets due to their location on the plasma membrane, where they can be easily accessed by drugs. The present review aims to provide a critical and up-to-date overview of NET development integrating Ca^{2+} signal involvement. In this perspective, we first give an introduction to NET and Ca^{2+} channels and then describe the different families of Ca^{2+} channels implicated in NET, i.e., ionotropic receptors, voltage-dependent Ca^{2+} channels, and transient receptor potential channels, as well as intracellular Ca^{2+} channels and their signaling molecules.

Introduction

Neuroendocrine tumors (NET) represent a variety of malignancies with a heterogeneous histology. They originate in neuroendocrine cells located all over the body [1]. The term neuroendocrine refers to the "neuro" (identified as presence of dense core granules) and "endocrine" properties (identified as the ability to secrete monoamine) of the cells composing the mass. NET are a relatively low-incidence disease (about 0.5% of the total estimated diagnosed tumors) but exhibit an increasing frequency and prevalence, with the most common being gastroenteropancreatic (GEP), bronchopulmonary (pNET), and thymus tumors and several uncommon localizations such as

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the ovaries, heart, and ear [2, 3]. Although their classification is quite confusing, in general NET can be categorized into the following 2 different groups based on clinical behavior, histology, and proliferation rate: low-grade indolent tumors (well-differentiated cells) and high-grade aggressive carcinomas (poorly differentiated cells).

Well-differentiated NET express typical neuroendocrine markers such as chromogranin A (CgA) and synaptophysin (Syn); on the contrary, poorly differentiated NET cells present a sheet-like proliferation more typical of carcinomas with limited immunocytochemical staining patterns for neuroendocrine markers (diffuse expression of Syn and faint or focal staining for CgA). Up to 40% of NET contain elements of a nonneuroendocrine histology; by definition, the neuroendocrine component has to exceed 30% of the tumor to be called a NET; otherwise, it is classified as a mixed adenoneuroendocrine carcinoma [2]. Other useful markers are somatostatin receptors (SSR), whose identification and quantification by immunohistochemistry or imaging are very useful to identify and predict the response to somatostatin analogs [3].

The general treatment for low-grade tumors is surgical resection, while unresectable and symptomatic disease is treated with somatostatin analogs and/or interferon-α even though tumor regression with these agents is rare [2, 4, 5]. In contrast, etoposide/platinum-based chemotherapy is the mainstay of treatment for high-grade or metastatic NET. As an alternative, peptide receptor radionucleotide therapy has been recently approved both in Europe and in US. Peptide receptor radionucleotide therapy relies on the use of somatoreceptor ligand conjugated with radioactive isotopes such as yttrium-90 and/or lutetium-177 for treatment purposes [6]. NET are also highly vascularized, and thus angiogenesis inhibitors such as sunitinib or VEGF inhibitors are good candidates for treatment [2].

Despite great efforts in recent years, the management of NET is still a critical unmet need due to the lack of knowledge of the biology of the disease, the lack of adequate biomarkers allowing identification of the primary tumor site or differentiation of tumor grading, late presentation, the relative insensitivity of imaging modalities, and a paucity of predictably effective treatment options [3].

Ca^{2+} Homeostasis Deregulation in Cancer

Accumulating evidence demonstrates that the development of several cancers, including NET, involves an altered Ca^{2+} homeostasis and aberrant ion channel expression [7, 8]. This is not surprising considering the multifaceted role of Ca^{2+} as a ubiquitous second messenger which is involved in the tuning of multiple fundamental cellular functions [9]. It has to be considered that the ubiquity of Ca^{2+} signals is not in antithesis with a specific role in a particular oncogenic mechanism. Each cell indeed possesses a Ca^{2+} machinery that enables the activation of Ca^{2+} signals of a particular amplitude, frequency and intracellular location. The presence of particular fingerprints allows Ca^{2+} to control specific cellular functions that may be altered during cancer progression [7, 10]. The intracellular Ca^{2+} concentration, i.e., $[Ca^{2+}]i$, is finely regulated and the different mechanisms involved in Ca^{2+} homeostasis are usually referred to as a "Ca^{2+} toolkit" and include Ca^{2+}-permeable channels, pumps, and exchangers [11]. The concentration gradient between intracellular cytosolic free $[Ca^{2+}]$ (~100 nM) and Ca^{2+} in extracellular fluids (~1 mM) is very large compared to those of other ions, being about 1:10,000. This gradient is assured by several "on" and "off" mechanisms that finally result in Ca^{2+} signals that can be codified in amplitude and frequencies. With regard to the "on" mechanisms, $[Ca^{2+}]i$ can increase via the following 2 different mechanisms: release form intracellular stores (mainly ER but also mitochondria or endolysosomes as an example) or entry from an extracellular medium via Ca^{2+}-permeable ion channels opened on the plasma membrane thanks to the strong electrochemical gradient that promotes the influx of Ca^{2+} into the cell [11].

Ion channels therefore represent a good potential pharmacological target also due to their location on the plasma membrane, where they can be easily accessed by drugs. Since the first reports identifying the role of ion channels in cancer development [12–15], the field has undergone exponential development, giving rise to a large consensus in the scientific community to consider ion channels in cancer development as "oncochannelopathy" [16, 17]. Beside ion channels, altered Ca^{2+}-regulated proteins have been also extensively investigated as a possible target to modulate cancer development [7].

A general classification of Ca^{2+} channels can be described on the basis of the gating mechanism. In this context the studies of electrical excitability of the 1950s and 1960s provided a good basis for classification into voltage-gated channels and ligand-gated channels. Voltage-gated Ca^{2+} channels comprise 3 voltage-gated calcium channel subfamilies, i.e., CaV1, CaV2, and CaV3, encoded by 10 genes [18]. The ligand-gated channel classification can be achieved according to the nature of the signaling molecule (ligand) that activated them (e.g., acetylcho-

line, glutamate, serotonin, or ATP). In recent years, intense studies have led to an exponential increase in the number of ion channel types and families thanks to the application of molecular biology techniques to the cloning of their gene [16]. In particular the cloning of transient receptor potential (TRP) ion channels gave rise to a whole new family of channels which are good candidates for mediated non-voltage gated Ca^{2+} signals. TRP channels include ion channels with a high selectivity for Ca^{2+} and potential constitutive activity (e.g., TRPV5 and TRPV6), as well as temperature-sensitive channels such as the cold sensor TRPM8 and the heat and capsaicin (hot chilly component)-sensitive TRPV1 [19]; different TRP channels are activated by second messengers and can promote a Ca^{2+} influx via store-operated Ca^{2+} entry (SOCE), which is activated in response to endoplasmic reticulum (ER) Ca^{2+} store depletion. In physiological conditions this is achieved by agonist-stimulated inositol 1,4,5-trisphosphate (IP3) generation and release of ER Ca^{2+} through the IP3 receptor (IP3R). In turn, Ca^{2+} release is detected by the ER Ca^{2+} sensor stromal interaction molecule 1 (STIM1). Upon Ca^{2+} store depletion, STIM1 proteins form clusters and subsequently interact with TRP channels proteins found at the plasma membrane, leading to activation of a Ca^{2+} influx [20]. Another important Ca^{2+} channel is calcium release-activated calcium channel protein 1 (ORAI1), which is involved in SOCE [21]. In particular, strong evidence has shown that, besides STIM1, also STIM2 is involved in ORAI1 activation under low-agonist, low-ER Ca^{2+} release by promoting STIM1 clustering in ER-PM junctions and thus increasing the assembly of the ORAI1-STIM1 complex and activation of SOCE [22, 23]. ORAI family of channels comprise another 2 related proteins, i.e., ORAI2 and ORAI3, which do not mediate SOCE (NSOCE) Ca^{2+} signals as homotetramers but can mediate SOCE Ca^{2+} signals in heteromeric channels composed of ORAI isoforms particularly in neurons where ORAI2 is likely to be the main candidate for SOCE [24–26].

In order to categorize all of the channels expressed at a cellular level, the term "channelome" has been reported in analogy with the widely accepted terms "genome," "proteome," and "metabolome," and the branch of research focused on the study of ion channelomes has been referred to as "channelomic" [16].

In this regard identification of the channelome in specific cancer types is especially important, since its determination might help to set up strategies to specifically target cancer cells but not healthy tissues, in contrast to the most widely used chemotherapics which affect the most rapidly proliferating cells. In addition, the tissue-specific location of these channels and their variable structure could render the treatment of oncochannelopathies possible without causing considerable side effects to other organs (i.e., liver, kidney, central nervous system, medulla etc.).

Ionotropic Receptors and NET Progression: *N*-Methyl-D-Aspartate Receptor

Ionotropic receptors are highly expressed and play key roles in different crucial aspects of the neuroendocrine cell physiology, ranging from excitation to synaptic release and gene expression. It is therefore not surprising that alteration of their function is involved in several hallmarks of NET progression.

Among the ionotropic receptors, *N*-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor present in most excitatory neuronal synapses where it modulates synaptic plasticity with peculiar roles in learning and memory as well as neuron maturation. NMDAR is a nonselective Ca^{2+}-permeable channel also depicted as a "coincidence receptor" due to its voltage-dependent inhibition by Mg^{2+} [27]. The expression and functional activity of NMDAR has been reported in different cancer tissue and cell types such as small-cell lung cancer and breast cancer [28–30] or prostate cancer [31]. More recently, different research papers have implicated NMDAR in pancreatic neuroendocrine cancer (PNET) in vivo as well as in vitro [32]. The authors showed that NMDAR is upregulated in the periphery of PNET tumors, both in an Rip1-Tag2 mouse model and in human tissue microarrays [33]. Inhibition or downregulation of NMADR but not AMPAR, another glutamate ionotropic receptor, significantly inhibits cell invasion. Interestingly, NMADR is associated with vGlut family proteins, i.e., vesicular glutamate transporters that export glutamate in the presynaptic membrane to initiate the signals by activating postsynaptic membrane. The authors postulated that in PNET autocrine glutamate secretion is involved in their capability for invasion. More recently, this hypothesis has been strengthened by electrophysiological inhibition of autocrine-activated NMDAR activity by vGlut inhibitor treatment [32]. The activation of NMDAR signaling is followed by increased phosphorylation of calmodulin kinase type II and calmodulin kinase type IV, leading to a modest increase in CREB phosphorylation at Ser133. These data together with BAPTA-AM inhibition of NMDAR-mediated invasion, clearly showed a central

role of Ca^{2+} signaling in the process [33]. Interestingly Hanahan et al. [33] proposed an intriguing mechanism by which NMDAR in PNET cancer cells can hijack the glutamate-NMDAR signaling normally used by neurons to promote cell invasion via a cancer-specific mechanism. The authors hypothesized that the high interstitial fluid pressure (typical of solid tumors) and the consequent pressure drop at tumor margins activate glutamate release via a mechanosensory pathway. In turn, glutamate release promotes NMDAR activation with consequent intracellular Ca^{2+}-mediated signal transduction that promotes cell invasion.

Voltage-Gated Ca^{2+} Channels and NET Progression

A close correlation between voltage-gated Ca^{2+} channels and neurendocrine differentiation (NED) has been extensively observed in prostate cancer [34, 35]. Indeed, the presence of neuroendocrine markers like CgA is correlated with prostate cancer dedifferentiation [36] and the presence of neuroendocrine cells in prostate cancer is correlated with a negative prognosis [37]. This is mainly due to the secretion of many neuropeptides with mitogenic activities like parathyroid hormone-related peptides, calcitonin, or gastrin-related peptides by neuroendocrine prostate cells which in turn could be responsible for the progression of cancer toward an androgen-independent stage. In this context, neuroendocrine prostate cancer cells overexpress a voltage-dependent calcium current of the T-type family, and in particular the channel subunit involved in this calcium current was shown to be the CaV3.2 (α1H) pore subunit [34]. This overexpression is attributable to upregulation of early growth response 1 and downregulation of repressor element (RE)-1- silencing transcription factor, which positively and negatively regulate transcriptional expression of Cav3.2, respectively [38]. Functional expression of CaV3.2 (α1H) sustains morphological differentiation and survival of neuroendocrine differentiated cells [39, 40]. Besides sustaining the NED of prostate cells, Ca^{2+} signals activated by ionomycin or thapsigargin treatments significantly promote secretion of prostatic acid phosphatase from NED. The specific role of CaV3.2 (α1H) was demonstrated by means of both pharmacological inhibitors or siRNA specifically directed against the channel. Both approaches show a clear involvement of CaV3.2 (α1H) in promoting both synthesis and secretion of prostatic acid phosphatase, and most likely serotonin, therefore being responsible of an enhanced autocrine/paracrine secretion in neuroendocrine prostate cancer. This phenomenon has been suggested to be in turn responsible for the progression of prostate cancer toward an androgen-independent stage [35]. Because of their lower threshold for activation, T-type Ca^{2+} channel activity can be significant at membrane potentials close to rest, resulting in a "window current" and consequent basal Ca^{2+} entry that is likely to be responsible for the neuropeptide secretion, explaining the role of these channels even in the absence of action potential [35].

PNET cells (BON) express a different subset of voltage-gated Ca^{2+} channels which are involved in CgA release in BON cells or insulin release from insulinoma INS cell lines. In both cases the secretion relies in fact on R-type Cav (CaV 2.3). This suggests a critical role in certain clinical characteristics of NET, such as hypersecretion syndrome [41]. Interestingly, CaV2.3 are also involved in somatostatin-mediated inhibitory mechanism of insulin release in pancreatic β cells. The activation of somatostatin receptor 2 significantly decreases CaV2.3-mediated Ca^{2+} signals with a consequent inhibition of insulin release [42].

TRP Channels NET

The TRP family of channels encompasses 27 members of non-voltage-gated cation channels that are Ca^{2+} permeable though not selective for most of them [43]. Several TRP channels are deregulated in cancer cells and have been suggested as valuable markers in predicting cancer progression and as potential targets for pharmaceutical therapy [7, 44]. Concerning NET, TRP channels have been involved in neurosecretion and cell proliferation and in particular TRPM8, TRPV1, and TRPV6 in PNET BON cells lines as well as in primary PNET cells [45–47].

Cold/menthol-sensitive TRPM8 activation by icilin elicits $[Ca^{2+}]i$ increases and secretion of neurotensin (NT) in BON cells as well as in primary PNET [45]. Interestingly NT is not expressed in the healthy pancreas, while its expression and secretion could be switched on during tumorigenesis of pancreatic endocrine cells. The release of NT could have a double physiopathological role: on one side NT is a potent stimulator for a number of secretion processes of the gastrointestinal tract and increased local and systemic levels of NT may contribute to hypersecretion characteristic of the carcinoid phenotype. On the other hand, NT have also been shown to sustain cell proliferation and enhanced tumor growth in different

in vitro and in vivo studies [48]. However, how the channel activation induces NT secretion should be further clarified. TRPM8 has been previously reported to participate in secretory pathways, in particular in cold-induced mucus hypersecretion of bronchial epithelial cells [49]. TRPM8-mediated airway mucus hypersecretion is induced by cold air in airway epithelial cells through the Ca^{2+}-PLC-PIP2-PKC-MARCKS signaling pathway. A secretory function has also been suggested for this channel in the prostate due to its localization in the epithelial cells on the apical side of the prostatic acini [50]. Even though this hypothesis was not further demonstrated, TRPM8 high expression was correlated with early prostate tumor progression while the channel expression decreases with tumor progression to the late, invasive, androgen-insensitive stage [for a review see 51]. In this context, it has emerged as an important factor in cell migration and prostate cancer progression, showing a protective role in metastatic prostate cancer due to its inhibition of cancer cell migration [52–55]. On the other hand, the short TRPM8 (sM8–18) isoform seems to have an opposite role, leading to an increase in prostate cancer cell migration and invasiveness through the activation of MMP-2 [56]. This effect could also result from sM8–18 inhibition of full-length TRPM8, as we have shown that the channel negatively regulates migration even if the interaction between short isoforms and ER-located TRPM8 has not yet been confirmed. In this respect, given the aggressiveness of NET it would be interesting to investigate which isoforms are expressed in NET and whether they affect cell migration in addition to NT secretion.

Beside TRPM8, TRPV1 is also implicated in neurosecretion in BON-1 PNET cells. In particular TRPV1 activation by capsaicin promotes CgA secretion, a common marker indicating hormone neuropeptide and biogene amine release [57]. TRPV1-mediated CgA release could have some potential relevance for PNET physiopathology, considering the fact that TRPV1 activity is regulated by somatostatins which inhibit also CgA release [58]. It is easy to speculate that TRPV1 could be one of the mechanisms involved in CgA secretion as a target of somatostatin activity, which is an important therapeutic target for NET.

Finally, TRPV6 is expressed in several PNET cells, including BON-1, where it controls Ca^{2+} homeostasis. The presence of TRPV6 in neuroendocrine cancer cells mediates cell proliferation; TRPV6 downregulation reduces cell growth by approximately 30% and leads to a declined CCND1 and CDK4 expression without affecting CCND2. TRPV6-mediated cell proliferation is dependent on NFAT-Ca^{2+} activation as shown by TRPV6 downregulation [47]. These data are in accordance with a previously reported role of TRPV6 in both prostate and breast cancer proliferation and growth. In particular TRPV6-mediated prostate cancer cell proliferation implicates NFAT activation [59].

Intracellular Ca^{2+} Signals in NET

The last paragraph of the present review is dedicated to a discussion of the role of intracellular Ca^{2+} signals in NET. We will analyze in particular the role of specific Ca^{2+} entry mechanisms triggered by the release from intracellular stores giving rise to SOCE or alternatively NSOCE mechanisms [11].

The role of Ca^{2+} homeostasis in NED has been clearly established in prostate cancer. As reported in previous paragraphs, in vitro NED differentiation is a poor prognosis marker in prostate cancer progression and it is frequently associated with androgen-independent states of cancer [37]. In vitro NED differentiation of the epithelial prostate LnCaP cell line induced by androgen depletion or [cAMP] increase causes marked changes in Ca^{2+} homeostasis including reduced filling of the ER Ca^{2+} store, decreased expression of both endolemmal SERCA 2b Ca^{2+} ATPase and the luminal Ca^{2+} binding/storage chaperone calreticulin, and a substantial downregulation of SOC current (I_{SOC}) [60]. The reduction of SOCE is due to cytoskeleton reorganization, especially F-actin over-polymerization [61]. As a final effect, SOCE downregulation in NE cells is involved in an increase in thapsigargin (Tg) or TNFa apoptosis resistance [60].

SOCE activation has also been described in different cell lines of GEP NET (GPNET). In particular SOCE-mediated Ca^{2+} signals can be recorded by exogenous store depletion by means of cyclopiazonic acid or muscarinic receptor activation by carbachol and are significantly inhibited by Gd3+ 1uM or BTP-2 perfusion. However, the authors did not describe any cellular function associated with SOCE [62].

Interestingly, recently NSOCE-mediated Ca^{2+} signals have been described in the GPNET cell line BON-1 after exogenous application of arachidonic acid (AA). Form the molecular point of view, both ORAI1 and ORAI3 channels are required for AA-mediated Ca^{2+} signals. However, ORAI1 was necessary for mediation of SOCE, whereas ORAI1 and ORAI3 were both required for AA-induced Ca^{2+} entry as well as BON cell migration. Moreover, the activation of NSOCE by AA correlates with an

increase in BON-1 cell migration as well as induction of neuroendocrine mesenchymal transition [63]. These data are in accordance with previously reports showing that AA is a key player in cell migration [64, 65].

Conclusions

Even though few Ca^{2+} channels and their signaling molecules have been shown to be implicated in NET, they have constituted a growing field of interest in recent decades. Indeed, the expression of several channels from different families (ionotropic receptors and voltage-gated, TRP, and SOCE components) were shown to be deregulated in NET, thus mainly affecting neurosecretion, cell proliferation, neuroendocrine cell differentiation, and invasion mostly in GPNET. Interestingly the drivers of this deregulation are not the same for all channels analyzed. The role in NET progression is associated either with increased expression or with specific activation due to a peculiar tumor microenvironment. In this context, the link between a high intracellular interstitial pressure and a mechano-activated glutamate release, which in turn promotes NMDAR activation and Ca^{2+}-mediated cell invasion, as proposed by Hanahn et al. [33], is in fact quite intriguing. Therefore, the peculiar physical characteristics of the tumor microenvironment are responsible for NMDAR-specific activity in cancer cells. On the other hand, regarding the role of T-type Ca^{2+} channels in neuroendocrine prostate cancer cells, Mariot and colleagues described an upregulation of the CaV3.2 channel subunit αH1 in neuroendocrine differentiated LNCaP as compared with control (not differentiated) LNCaP [35]. Moreover, the marked changes in Ca^{2+} homeostasis observed in NED-differentiated LNCaP cells is due to cytoskeleton reorganization, especially F-actin overpolymerization as discussed by Prevarskaya and colleagues [61].

It would be interesting, though, to further characterize the molecular mechanisms underlying these cellular functions to carcinogenesis and investigate putative consequences in Ca^{2+} homeostasis. In this perspective, there have been several cases reports linking NET in the thymus, pancreas, and gastrointestinal tract to hypercalcemia [66, 67], suggesting that excessive hormone secretion influences Ca^{2+} channels and their signaling pathway. Increased serum Ca^{2+} levels in NET were mostly correlated with high 1,25-dihydroxyvitamin D and parathyroid hormone-related protein (PTH-rP) secretion. This link has to be further investigated since it is well known that 1,25-dihydroxyvitamin D and PTH regulate Ca^{2+} channel expression and in particular that of TRPV5 and TRPV6, both of which are involved in Ca^{2+} intestinal absorption, distal tubular reabsorption, and bone resorption [68].

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflict of interests to declare.

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