Ca2+ channels toolkit in Neuroendocrine tumors

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Abstract

Neuroendocrine tumors (NET) constitute an heterogenous group of malignancies with various clinical presentations and growth rates but all rising from neuroendocrine cells located all over the body. NET present a relatively low frequency disease being mostly represented by gastroenteropancreatic (GEP) and bronchopulmonary tumors (pNET); on the other hand an increasing frequency and prevalence has been associated to NET. Beside the great effort of the latest years, management of NET is still a critical unmet point due to the lack in the knowledge of the biology of the disease, 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 pro-angiogenetic 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 on NET development integrating Ca^{2+} signals 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, namely ionotropic receptors, voltage-dependent Ca^{2+} channels, Transient Receptor Potential channels as well as intracellular Ca^{2+} channels and their signaling molecules.

Introduction

Neuroendocrine tumors (NET) represent a group of a variety of malignancies with an heterogeneous histology. They rise from 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 ability to secrete monoamine) of the cells composing the mass. NET is a relatively low incidence disease (about 0.5% of the total estimated diagnosed tumors) but have exhibited an increasing frequency and prevalence, with the most common being gastroenteropancreatic (GEP), bronchopulmonary (pNET), thymus tumors and several uncommon localizations such as ovaries, heart and ear [2,3]. Although classification is quite confusing, in general NET can be classified into two different groups based on clinical behavior, histology, and proliferation rate: low grade indolent tumors (well differentiated cells) versus 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, faint or focal staining for CgA). Up to 40% of NET contain elements of non-neuroendocrine histology; by definition, the neuroendocrine component has to exceed 30% of the tumor to be called NET; otherwise, it is classified as a mixed adenoneuroendocrine carcinoma [2]. Other useful markers are the

somatostatins receptors (SSR) whose identification and quantification by immunohistochemistry or imaging are very useful to identify and predict the response to somatostatins 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 neuroendocrine tumors (NET). As an alternative, Peptide Receptor Radionucleotide Therapy (PRRT) has been recently approved both in Europe and in US. PRRT relies on the use of somatoreceptors ligand conjugated with radioactive isotopes such as yttrium-90 and/or lutetium-177 for treatment purposes [6]. NET are also highly vascularized thus angiogenesis inhibitors such as sunitinib or VEGF inhibitors are good candidates for treatment [2].

Beside the great effort of the latest years, management of NET is still a critical unmet point due to the lack in the knowledge of the biology of the disease, lack of adequate biomarkers allowing to identify the primary tumor site or to differentiate 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 altered Ca^{2+} homeostasis and aberrant ion channel expression [7,8]. This is not surprising considering the multifaceted role of Ca^{2+} as an 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 on a particular oncogenic mechanism. Each cell possesses indeed a Ca^{2+} machinery that enables the activation of Ca^{2+} signals of 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]. Intracellular Ca^{2+} concentration, $[Ca^{2+}]i$, is finely regulated and the different mechanisms involved in Ca^{2+} homeostasis are usually referred to as "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 as compared with other ions, being about 1:10000. This gradient is assured by several "ON" and "OFF"

mechanisms that finally results in Ca^{2+} signals that can be codified in amplitude and frequencies. As regarding the “ON” mechanisms, $[Ca^{2+}]i$ can increase via two different mechanisms: release form intracellular stores (mainly ER, but also mitochondria or endolysosomes as an example) or entry from 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 represent therefore a good potential pharmacological target due also 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 an 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 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 1950s an 60s provided good basis for classification in Voltage-gated channels (VGC) and Ligand-gated channels (LGC). Voltage gated Ca^{2+} channels are comprehensive of three voltage-gated calcium channel subfamilies: CaV1; CaV2; and CaV3 encoded by ten genes [18]. Within the LGC classification can be achieved according to the nature of signaling molecule (ligand), which activated them (e.g., acetylcholine, glutamate, serotonin, ATP). In more recent years, intense studies 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 high selectivity for Ca^{2+} and potential constitutive activity (for example, 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 Ca^{2+} influx via store operated Ca^{2+} entry (SOCE) which is activated in response to endoplasmic reticulum (ER) Ca^{2+} stores depletion. In physiological conditions this is achived 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 protein forms

clusters and subsequently interact with TRP channels proteins found at the plasma membrane, leading to activation of Ca^{2+} influx [20]. Another important Ca^{2+} channel is calcium release activated calcium channel protein 1 (ORAI1), involved in SOCE [21]. In particular strong evidences show that beside 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 assembly of the ORAI1-STIM1 complex and activation of SOCE [22,23]. ORAI family of channels comprehend other two related proteins ORAI2 and ORAI3, which mediates not SOCE (NSOCE) Ca^{2+} signals as homotetramers but can mediate SOCE Ca^{2+} signals in heteromeric channels composed of ORAI isoforms particulary in neurons where ORAI2 is likely to be the most candidate for SOCE [24–26].

In order to categorize all the channels set expressed at cellular level, the term “channelome” has been reported in analogy with the widely accepted “genome”, “proteome” and “metabolome” and the branch of research focused on the study of ion “channelome” 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 (liver, kidney, central nervous system, medulla etc).

Ionotropic receptors and NET progression: NMDAR

Ionotropic receptors are highly expressed and play key roles in different crucial aspects of neuroendocrine cell physiology ranging from excitation, 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 receptor, 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 non-selective Ca^{2+}-permeable channel also depicted as “coincidence receptor” due to its voltage-dependent inhibition by Mg^{2+} [27]. 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 implicated NMDAR in pancreatic neuroendocrine cancer (PNET) *in vivo* as well as *in vitro* [32]. The authors showed that NMDAR is upregulated at the periphery of PNET tumors, both in the Rip1-Tag2 mouse model as well as in human tissue microarrays [33]. Inhibition or downregulation of NMADR but not AMPAR, another glutamate ionotropic receptor, significantly inhibits cell invasion. Interestingly NMADR is associate to vGlut family proteins, vesicular Glutamate transporters that export Glutamate in 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 showing the electrophysiological inhibition of autocrine-activated NMDAR activity by vGlut inhibitor treatment [32]. The activation of NMDAR signaling is followed by an increased phosphorylation of calmodulin kinase type II (CaMK-II) and calmodulin kinase type IV (CaMK-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 Hanan and collegues proposed an interesting mechanism by which NMDAR in PNET cancer cells can hijak the glutamate-NMDAR signaling normally used by neurons to promote cell invasion by 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, activates glutamate release via mechano-sensory pathway. In turn glutamate release promotes NMDAR activation with consequent intracellular Ca^{2+}-mediated signal transduction that promotes cell invasion [33].

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 to a negative prognosis [37]. This is mainly due to the secretion of many neuropeptides with mitogenic activities like parathyroid hormone-related peptide, 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 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 (Egr-1) and downregulation of repressor element (RE)-1- silencing transcription factor (REST), that 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]. Beside sustaining the NED of prostate cells, Ca^{2+} signals activated by iononomycin or thapsigargin treatments significantly promote secretion of prostatic acid phosphatase (PAP) form NED. The specific role for 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 as well as secretion of PAP, and most likely serotonin, therefore being responsible of an enhance 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, from 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].

Pancreatic neuroendocrine cancer 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 suggest a critical role in certain clinical characteristics of NET, such as the hypersecretion syndrome [41]. Interestingly CaV2.3 are also involved in somatostatins-mediated inhibitory mechanism of insulin release in pancreatic ß cells. Activation of somatostatins receptors 2 decrease significantly CaV2.3-mediated Ca^{2+} signals with a consequence inhibition of Insulin release [42].

TRP channels NET

The TRP family of channels encompass 27 members of non voltage-gated cation channels Ca^{2+}-permeable although not selective for most of them [43]. Several TRP channels result de-regulated 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 pancreatic NET 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 pancreatic NET [45]. Interestingly NT is not expressed in 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 also sustain cell proliferation and enhanced tumor growth in different *in vitro and in vivo studies* [48]. However, it should be further clarified how the channel activation induces NT secretion. 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 prostate due to its localization in the epithelial cells at 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 channels 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 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 inhibiting 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 on BON-1 PNET cells. In particular TRPV1 activation by capsaicin promotes CgA secretion, a common marker indicating hormone neuropeptides and biogenes amines release [57]. TRPV1- mediated

CgA release could have some potential relevance on PNET physiopathology considering the fact that TRPV1 activity is regulated by somatostatins which inhibits also CgA release [58]. It is easy to speculate that TRPV1 could be one of the mechanisms involved in CgA secretion as target of somatostatins activity, which are important therapeutic targets for NET. Finally, TRPV6 is expressed in several pancreatic NET 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 declined CCND1 and CDK4 expression, without affecting CCND2. TRPV6-mediated cell proliferation is dependent upon NFAT-Ca^{2+} activation as shown by TRPV6 downregulation [47]. These data are in accordance with previously reported role for 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 the 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 neuroendocrine differentiation 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 is frequently associated with androgen independent states of cancer [37]. *In vitro* NED differentiation of 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, the decreased expression of both endolemmal SERCA 2b Ca^{2+} ATPase and the luminal Ca^{2+} binding/storage chaperone calreticulin, as well as 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 final effect, SOCE downregulation in NE cells is involved in an increase of both thapsigargin (Tg) or TNFa apoptosis resistance [60].

SOCE activation has been also described in different cell lines of gastroenteropancreatic neuroendocrine tumors (GPNET). In particular SOCE-mediated Ca^{2+} signals can be recorded both by exogenous store depletion by means of cyclopiazonic acid (CPA) 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 GPNET cell lines 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 mediating SOCE, whereas Orai1 and Orai3 were both required for AA-induced Ca^{2+} entry as well as BON cell migration. Moreover 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 constitute a growing field of interest in last decades. Indeed, the expression of several channels from different families (ionotropic receptors, voltage-gated, TRP and SOCE components) were shown to be deregulated in NET, affecting thus mainly 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 to increased expression or specific activation due to peculiar tumor microenvironment. In this context, it is in fact quite intriguing the link between high intracellular interstitial pressure and mechano-activated Glutamate release which in turn promotes NMDAR activation and Ca^{2+} mediated cell invasion proposed by Hanahn and collegues [33]. It is therefore the peculiar physical characteristics of tumor microenvironment which is responsible for NMDAR specific activity in cancer cells. On the other hand, as regarding T-type Ca^{2+} channels role in neuroendocrine prostate cancer cells, Mariot and collegues described an upregulation of 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 over-polymerization as discussed by Prevarskaya and collegues [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

thymus, pancreas and gastrointestinal tract to hypercalcemia [66,67] suggesting that excessive hormones 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 the one of TRPV5 and TRPV6, both involved in Ca^{2+} intestinal absorption, distal tubular reabsorption and bone resorption [68].

Statements

Statement of Ethics

The authors have no ethical conflicts to disclose."

Disclosure Statement

The authors have no conflicts of interest to declare.

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