VOLTAGE-OPERATED AND NON-VOLTAGE-OPERATED Ca^{2+} ENTRY PATHWAYS IN GASTROENTEROPANCREATIC NEUROENDOCRINE TUMOR CELL LINES

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Introduction

The role for Ca^{2+} in cancer-related cell signaling pathways is well established. Alterations in Ca^{2+} homeostasis increase proliferation and induce differentiation and apoptosis. According to a growing number of studies, Ca^{2+} channels voltage- and non-voltage-gated family represents key players in calcium homeostasis and cell physiopathology (Montell, *et al*,. 2002, Prevarskaya, *et al*, 2007).

We proposed that Ca^{2+} entry through plasma membrane channels could provide an additional or alternative pathway for modulation of cell growth in gut neuroendocrine cells. To investigate this possibility, we characterized Ca^{2+} entry in a set of human carcinoid cell lines originating in the foregut, midgut and hindgut as a starting point for an inquiry into the role of Ca^{2+} signaling pathways in carcinoid cancer.

To test this hypothesis, we used RT-PCR to profile a variety of voltage-operated and non-voltage-operated Ca^{2+} permeable channels and then characterized VOCE and SOCE in the carcinoid cell lines.

VOCC expression profiling and functional assessment

We first probed for ten VOCC $\alpha 1$ subunits classified into three families ($CaV_{1\text{-}3}$) (Catterall 2000). The CaV_1 family conducts L-type Ca^{2+} currents, which have been shown in a variety of cell types to be involved in gene regulation and hormone secretion. The CaV_2 family conducts N-, P/Q-, and R-type Ca^{2+} currents, which have neurosecretory function. These channels are considered HVA channels. The CaV_3 family conducts T-type currents and are considered LVA channels.

In the current study, messages for all of the major classes of VOCCs were identified as shown in Fig.1. However not all of the cells exhibited functional channels. We found that BON cells exhibited HVA currents whereas the midgut carcinoids used in the study did not appear to express functional channels.

This observation is consistent with the identification of both a greater variety and higher levels of messages in foregut carcinoids compared to midgut and hindgut carcinoid lines and correlated with an assessment by Mergler (2003) who noted that primary cultures of foregut carcinoids exhibit larger Ca^{2+} currents than midgut carcinoids. We next demonstrated pharmacologically that L-type current is the predominant current in BON cells when elicited by K^+ depolarization or under voltage clamp. Alpha 1D isoform of L-type channel exhibits strong relative expression in foregut carcinoids, and is the major channel expressed in the midgut carcinoid lines (see Fig. 4). In BON cells, L-type and R-type channels are known to couple to exocytosis of peptides.

Whereas a variety of HVA Ca^{2+} channels including L-, N-, P/Q- and R-type have been identified in primary cell cultures from resected neuroendocrine gut tumors and in some human and murine carcinoid cell lines, there was little infor-

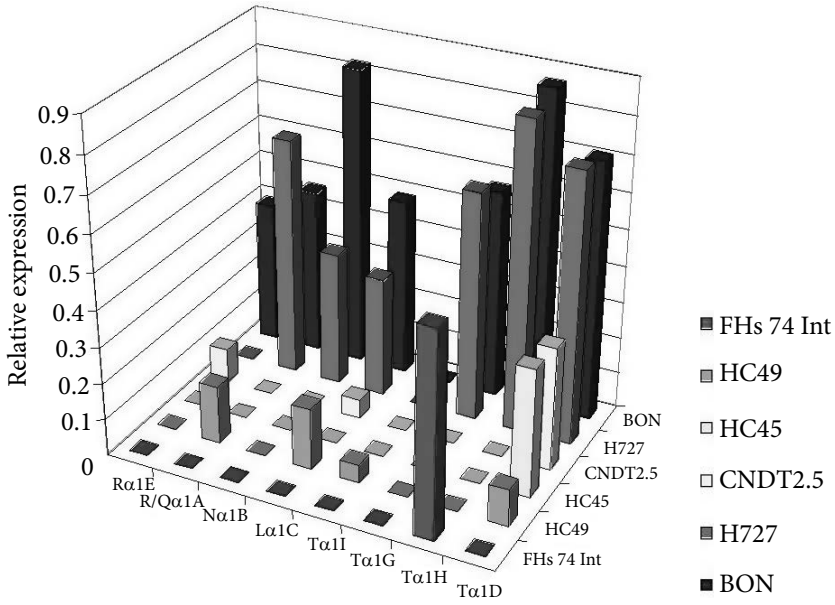

Fig. 1. Semi-quantitative endpoint RT-PCR of voltage-operated Ca^{2+} channel gene expression in carcinoid cell lines and the FHs 74 Int intestinal epithelium cell line. Gel band intensity values were determined by densitometry. Intensities were normalized to ß-actin gene expression and bars represent mean ratio values for at least 3 experiments

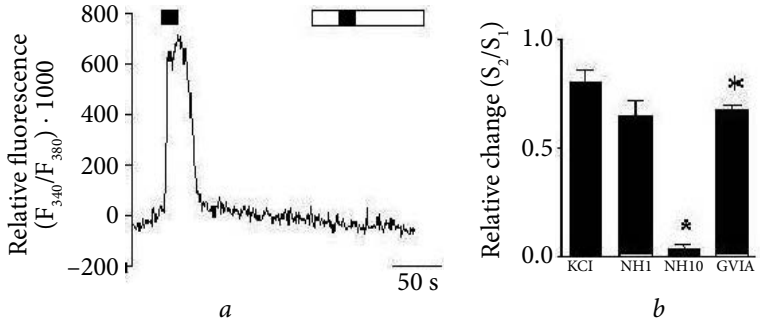

Fig. 2. Functional assessment of voltage dependent Ca^{2+} entry in single BON cell; *a* — Representative fura-2 traces showing Ca^{2+} signals evoked by successive 30 s bath application of physiological saline (PSS) containing 80 mM K^+ (black stimulus bars) prior to and following treatment with 10 μM nifedipine (open stimulus bar); *b* — Ratio values determined from S_2 (second stimulus) to S_1 (first stimulus) peak amplitudes of control (KCl), and treated

mation available regarding T-type currents in carcinoid tumor cells. T-type channels have been implicated in some transformed cell lines in the transition from epithelial to neuroendocrine phenotype and in proliferation (Jones, *et al,* 1998). T-type LVA channel subunits, α1H and α1G showed strong relative expression in

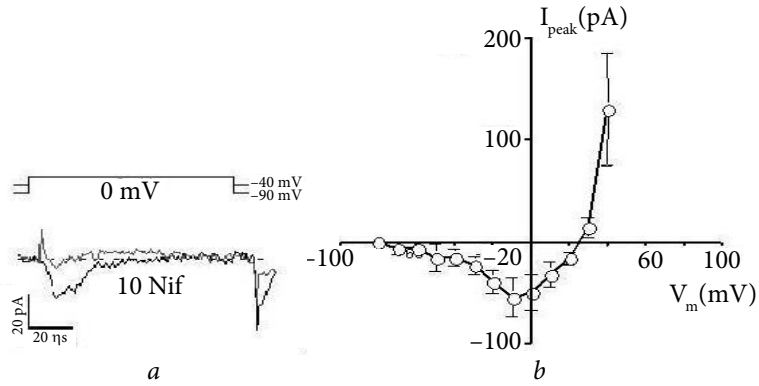

Fig. 3. Currents evoked during application of 10 μM nifedipine (Nif) and in control (*a*); *b* — Peak amplitudes for evoked currents were determined following random 10 mV increment step depolarizations from a HP –90 mV, averaged and plotted as a current-voltage relationship in BON cells (n = 3)

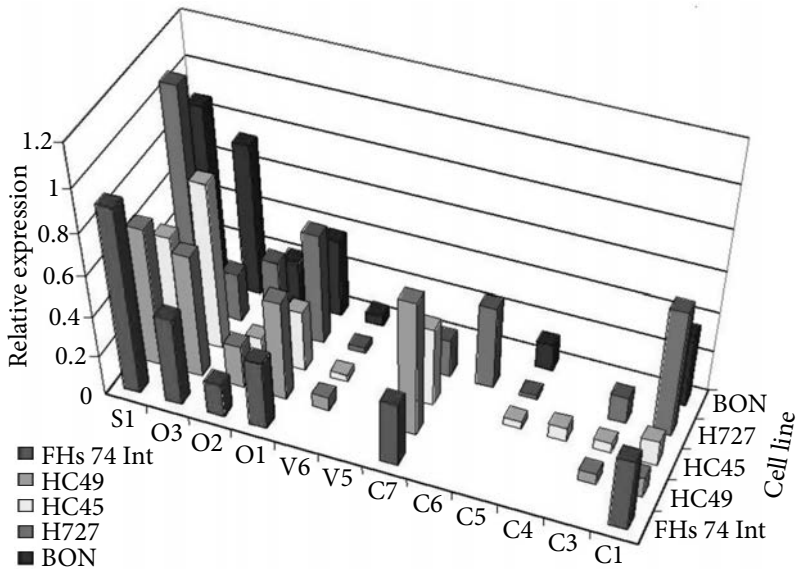

Fig. 4. Semi-quantitative RT-PCR of some non-voltage-operated, store-operated and store-independent $Ca^{\cdot}$ channels in a set of carcinoid cells and in FHs 74 Int cells. Gel band signal intensities were normalized to ß-actin signal. Mean ratio values represent result of at least independent 3 experiments

foregut carcinoids. Using selective voltage protocols and pharmacology, we functionally identified a putative T-type current in a subset of BON cells (Fig. 3). The current was activated at –70 mV, inactivated with a time constant of about 20 ms and blocked by 10 μM Ni^{2+}. R-type channels are also expressed in BON cells, and

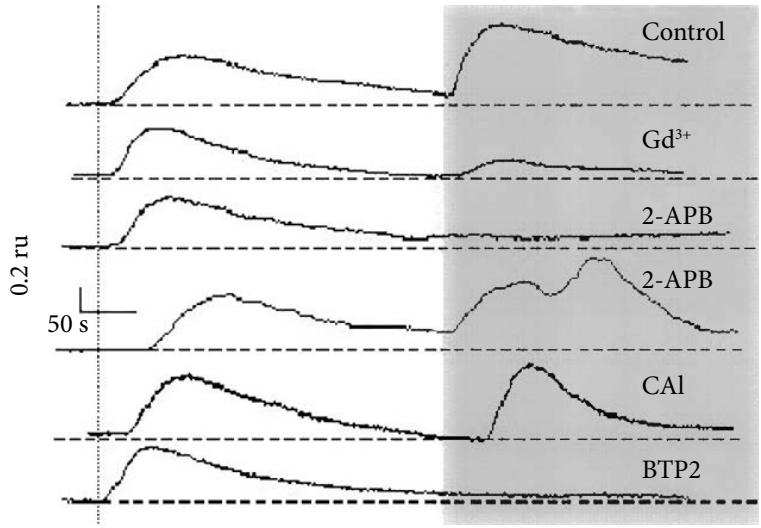

Fig. 5. Representative fura-2 traces showing effects of various channel inhibitors on SOCE following restoration of extracellular Ca^{2+} (shaded box)

like T-type channels, are activated at somewhat more negative voltage than other HVA channels, inactivated rapidly and are sensitive to be blocked by $NiCl_2$ (Jones, *et al,* 1998). However, R-type channels are typically blocked by Ni^{2+} in the 100 μM range in contrast to the high-affinity block by Ni^{2+}, which we observed. These data suggested that the α1H ($CaV_{3.2}$) channel, which is highly sensitive to low concentration Ni^{2+} blockade, is the dominant LVA current in BON cells.

The α1H ($CaV_{3.2}$) channel was identified in the non-transformed small intestine epithelial cell line FHs 74 Int. We were not able to identify T-type channels in the midgut carcinoid lines suggesting that these channels may be expressed as splice that were not detectable by our methods or not be expressed in midgut carcinoid lines. Some studies have implicated CpG island hypermethylation as a negative regulator of α1G T-type channel gene expression in colon and neuroendocrine cancers (Chan, *et al,* 2003), although this gene appears to be unmethylated in foregut, midgut, and hindgut carcinoid tumors. Whether this regulation applies to other T-type channel genes is not known. Although the α1G product was identified in foregut carcinoid cell lines, our data point to the role for the α1H ($CaV_{3.2}$) channel.

SOCE in carcinoid cell lines

In recent years it has become apparent that SOCE is a critical regulatory signal in many epithelial and other "non-excitable" cell types. In addition, there has been substantial focus on SOCE as a signal for transcriptional regulation, cell growth, and survival in metastatic cells including colon and prostate cancers (Kazerouni-

an *et al*, 2005). The best characterized form of SOCE is the Ca^{2+} release activated current (I_{CRAC}) of lymphocytes of which the structural determinants include the ER luminal Ca^{2+} sensor STIM1 and pore-forming subunits of Orai1.

As seen in Fig. 5, we have identified STIM1 and all three human Orai paralogues in the carcinoid cell lines. This observation may be particularly relevant as Orai1 and STIM1 have recently been shown to be critical for breast tumor cell migration and metastasis (Yang, *et al,* 2009). Generally, we found that the relative expression levels for these pore-forming subunits were Orai3 > Orai1 > Orai2. The identification of STIM1 and Orai1 was consistent with our functional data which indicate SOCE in carcinoid cells and mostly consistent with our pharmacological profiling of SOCE. For example, low concentrations of the blockers Gd^{3+}, 2APB, CAI, and BTP2 reduced entry in most carcinoid cell lines (Fig. 4), consistent with the reported sensitivity of SOCE.

As shown in Fig. 5, 2-APB treatment in BON cells induces a complex response. For example, it appeared that 2-APB enhanced entry. One interpretation of this observation was that some BON cells express more Orai3, which, in contrast to Orai1, can be activated by 2-APB. Interestingly, the anti-tumor compound CAI was effective at reducing entry in midgut and hindgut carcinoid lines but did not consistently block Ca^{2+} entry in the foregut cell lines (data not shown). This may point to CAI as a potential compound to target Ca^{2+} entry in ileal carincoids. The variability of these responses may reflect the limited selectivity of these inhibitors and/or the complexity of channel subunit expression and interactions. Certainly, without improving the selectivity of blockade using better pharmacological probes, it is difficult to draw conclusions regarding the identity of channels underlying SOCE in the various carcinoid cell lines. Thus, experiments using gene silencing and overexpression methods are in progress.

We also used RT-PCR to screen carcinoid cell lines for an array of TRP channels, we identified TRPC1-7, and TRPV6, TRPM5 and TRPM8. These findings suggested that they might play important roles in encoding sensory information and growth of signals in enteroendocrine cells of the gut epithelia or contribute to malignant phenotype in GI cancers.

TRPC1 is the best-characterized member of the TRPC protein subfamily and is linked to proliferation, cell migration, and apoptosis in intestinal epithelia and in prostate cancer (Marasa, *et al,* 2008). In the current study, TRPC1 was identified in all of the tested cell lines, and its relative expression level was the highest for the foregut carcinoid lines and lowest for the midgut carcinoids. The relationship of TRPC1 to SOCE in carcinoid cell lines has not been elucidated here. According to the works of others, it probably does not form functional CRAC channels as overexpression studies do not recapitulate the electrophysiological properties of I_{CRAC}

Whereas STIM1, Orai1-Orai3 and TRPC1 were ubiquitously expressed, the other TRP family members were expressed in some cell lines but not in theothers. For example, TRPC3 and TRPC5 were identified in foregut and midgut, or in

midgut and hindgut carcinoid lines, respectively. TRPC4 was detected exclusively in HC45 cells, and TRPC6 was detected exclusively in H727 cells. TRPC7 was detected in all cell lines except BON cells. The specific role for TRPC channels in enteroendocrine cells or in carcinoid tumors remain unknown and further elucidation of their function should become a priority.

In addition to canonical TRP family members, other TRP subfamily members were implicated in cancers. In this study, message corresponding to TRPV6, known to underlie vitamin D-regulated Ca^{2+} uptake in the intestine, was expressed at relatively low levels in all carcinoid cell lines tested, but was not identified in the small intestinal epithelial line FHs 74 Int. TRPV6 is highly Ca^{2+} selective and has been shown to potentiate Ca^{2+} dependent cell proliferation (Shwartz, *et al,* 2007). This is aberrantly expressed in a variety of human cancers (Zhuang *et al,* 2002). Whether or not vitamin D regulates TRPV6 expression, cell growth or other functions in carcinoid cell lines is not known. In addition to TRPV6, TRPV2 was detected in BON, H727, and CNDT2.5 cells and TRPV1 was detected in H727 cells. TRPM5, which is not Ca^{2+} permeable, and TRPM8 were detected in foregut and bronchial carcinoid cell lines (BON and H727). It is tempting to speculate that these channels may play specific roles in sensory transduction or secretory function in gut enteroendocrine cells.

Conclusions

Ca^{2+} oscillations can induce resting cells to reenter the cell cycle and promote chemokinesis, migration, and invasive activity. Typically, the maintenance of Ca^{2+} oscillations is dependent not only on Ca^{2+} release from internal stores but also on SOCE. This prolonged Ca^{2+} entry can activate a number of signaling pathways and has been shown to regulate transcription factors like NFAT and CREB (Lipskaja, *et a,l* 2004). Our findings lend credence to the idea that SOCE plays a role in the control of cell growth in enteroendocrine and carcinoid cell lines, but additional studies areneeded to pinpoint whether Ca^{2+} entry is linked to transcriptional regulation in carcinoids cells.

Thus, in this study we identified a number of putative Ca^{2+} entry pathways in a set of carcinoid cell lines originating from bronchial epithelium, foregut, midgut and hindgut using molecular and functional assays. Although the molecular and pharmacological profiling presented here is not exhaustive, our study suggests these pathways *might* be potential targets for development of new diagnostic tools or anticancer therapies and provides an important starting point for the further clarification of the role of Ca^{2+} pathways in enteroendocrine cell biology in health and disease.

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