Ca^{2+} Entry & Maintenance of Oscillatory Ca^{2+} Signals in Human Carcinoid Cell Lines

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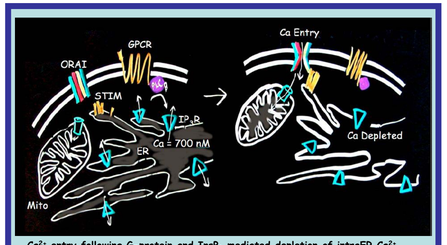
BACKGROUND

Calcium entry following endoplasmic reticulum (ER) Ca^{2+} depletion and store-operated Ca^{2+} channel activation regulates proliferation, migration and apoptosis in some cancer cells including those of neuroendocrine phenotype. We previously demonstrated that carcinoid cell lines express a variety of non-voltage operated Ca^{2+} channels including key components of store operated Ca^{2+} entry (SOCE): the ER Ca^{2+} sensor STIM1 and the plasma membrane channel proteins ORAI1 and ORAI3. However, the role of SOCE in carcinoid cell lines has not been elucidated. Thus to gain insight into STIM and ORAI function in foregut and midgut carcinoid cancers we used targeted gene silencing and over expression techniques in combination with live cell imaging of Ca^{2+} entry to assess the functional consequences of this Ca^{2+} entry pathway. This study points to a dominant role for STIM1 and ORAI1 in mediating SOCE in BON cells. Importantly, SOCE was required for the maintenance of GPCR-induced oscillatory Ca^{2+} signals, and ORA1 was shown to be critical for the establishment of tumor-like structures in an organ-slice model



Ca^{2+} entry following G-protein and $InsP_3$ mediated depletion of intraER Ca^{2+} is activated by the induced interaction between ER Ca^{2+} sensor STIM1 and pore-forming ORAI multimers.

METHODS

Cell Culture: Carcinoid cell lines were grown in DMEM 10% Serum and maintained at 37°C at 95%/5% O2/CO2.

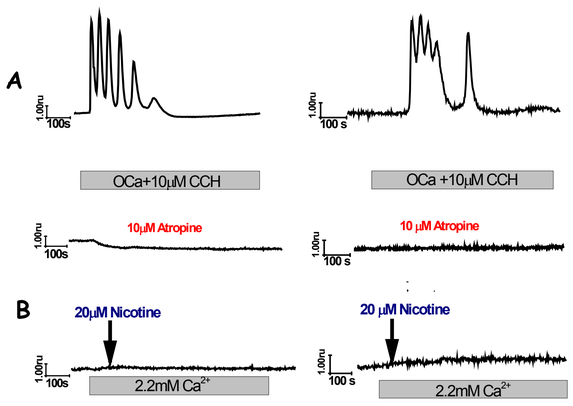
Calcium imaging: Intracellular calcium concentration was studied using Fura-2AM dye. The loaded cells were excited at two different wavelength and ratio of the emission gives the amount of Fura2 bound to calcium which indirectly reveals the intracellular calcium concentration. In some experiments Ca entry was measured using Mn quench or Ba influx methods.

Transfection: The shRNA, dominant negative and wild type constructs were introduced into carcinoid cell lines using Amaxa biosystems as described by manufacturer. Program T-20 was used to transfect the neuroendocrine cells. Estimated efficiency of transfection was approximately 60 -75%. Effectiveness of knockdown strategy was quantified by real-time RT-PCR.

Mutation: Site-Directed mutagenesis of Orai1E106A and Orai3E81A was performed as indicated in Quikchange II XL site -directed mutagenesis kit (Stratagene).

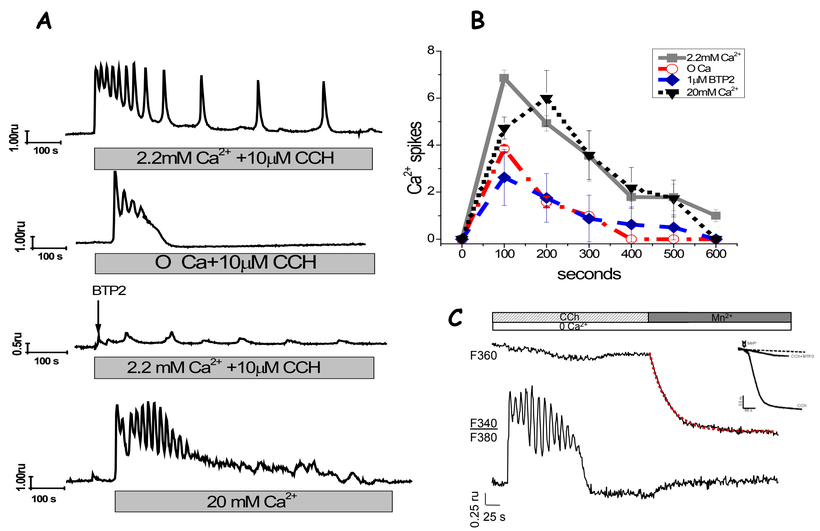
Organotypic liver slice: Hepatic portal circulation is a predicted route for establishing liver metastases of GEPNETs or following intra-splenic injection of cancer cells. We developed an organ slice model system to study human carcinoid cell dynamics ex vivo using mouse liver organotypic slice culture as a tractable preparation that more closely resembled a three dimensional, multi-cellular tumor microenvironment than dispersed cell culture systems. BON cells stably transfected with GFP were introduced to liver by portal vein injection. Vital stain of liver vasculature was achieved by co-injection with DiI-CM. Organotypic slices obtained from the liver by vibratome were monitored using fluorescence macroscopy. Slices (200 µm) were maintained in culture on nylon inserts and with RPMI for up to 2 weeks.

1. Carcinoid cell lines express mAchRs



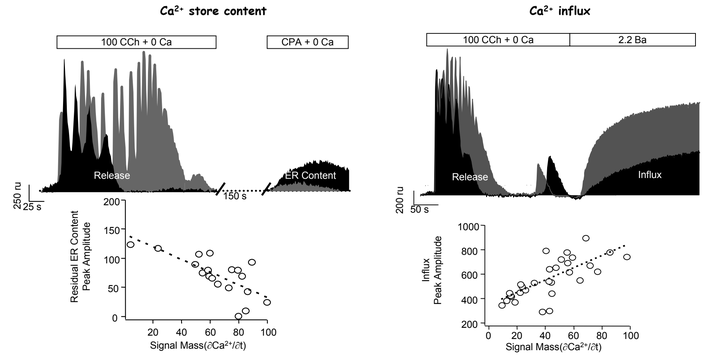
Representative traces for BON and H727 carcinoid cell lines. A. Examples of CCh-evoked Ca^{2+} oscillations and atropine inhibition of muscarinic receptors. B.Example traces showing lack of nicotinic receptor activity.

2. Muscarinic activation of Ca^{2+} entry (SOCE)



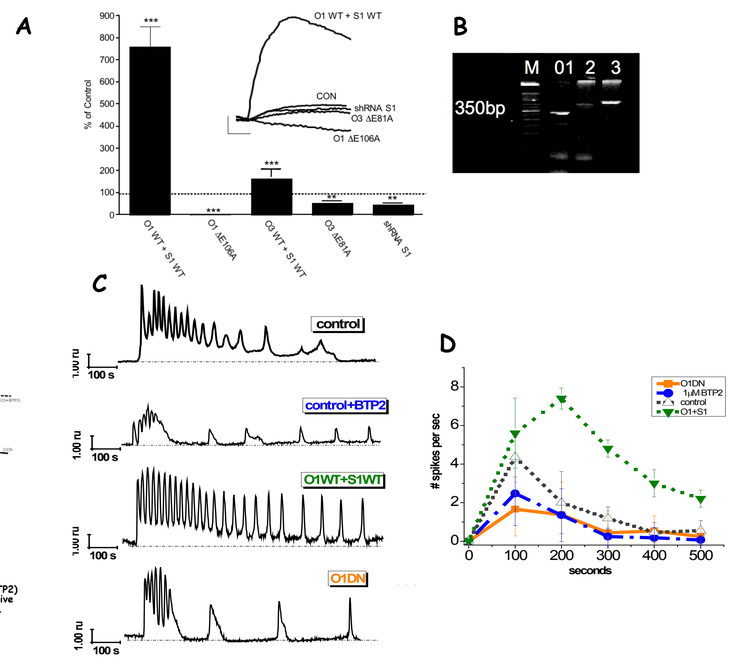
A. Representative traces of Ca^{2+} dynamics induced by CCh in the presence of various concentrations extracellular Ca^{2+} concentrations or SOCE inhibition (BTP2) B. The frequency of the oscillations under differing conditions was represented as plot of Ca^{2+} spikes vs time. C. Manganese assay of SOCE. A representative trace showing determination of magnitude and rate of influx by exponential fit (red colored trace) to the slope of Mn^{2+} induced fura-2 fluorescence quench. Inset: CCh evoked Ca^{2+} entry is blocked by the SOCE inhibitor BTP2.

3. Relationship of GPCR activation and SOCE



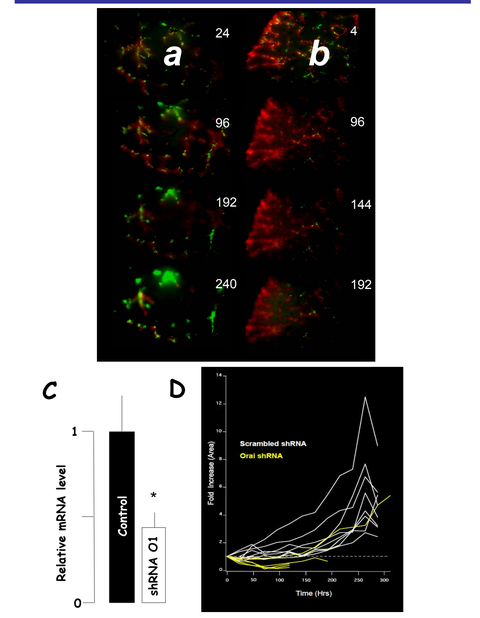
3. SOCE is related to the extent of GPCR activation and Ca^{2+} diminishment in ER. Inverse relationship of Ca store content (determined by CPA application) to the time integrated CCh-evoked Ca^{2+} release (Signal Mass). Direct relationship of magnitude of influx (measured by barium entry) to CCh-induced signal mass.

4. Role of ORAI in CCh-evoked entry and maintenance of Ca^{2+} oscillations



A.Functional assessment of SOCE by over-expression of wild type or dominant negative SOC proteins or by knockdown. B. Endpoint RT-PCR showing level endogenous ORAI proteins in BON cells.
C. Representative traces of Ca^{2+} dynamics induced by CCh in the presence or absence of BTP2 in BON cells over-expressing wild type or dominant negative ORAI1 (DN).
D. The frequency of the oscillations in CCh stimulated BON cells plotted as Ca^{2+} spikes vs time.

5. Tumor formation in mouse liver requires ORAI 1



a) Stably transfected BON cells expressing GFP and scrambled shRNA (control) proliferates in mouse liver as "tumorlets".b) In contrast ORAI1 knockdown BON cells do not.
c) Validation of of Orai1 stable knock down BON cells by quantitative real-time RT-PCR. Stable knockdown of Orai1 was achieved using shRNA.
d) Time course of tumor area fold increase in control (white) and Orai1 knockdown (yellow).

CONCLUSIONS

- mAchR activation in BON and H727 cells evoked Ca^{2+} oscillations in a dose- and extracellular Ca^{2+} dependent fashion.
- Inhibition of Ca^{2+} entry, silencing of ORAI or STIM or over-expression of a dominant negative ORAI significantly diminished Ca^{2+} oscillations, whereas over-expression of wild-type ORAI protein enhanced Ca^{2+} oscillations.
- In addition, BON cells deficient in ORAI were unable to reliably form tumors in our organ slice model.
- These data indicated that ORAI is required for agonist induced Ca^{2+} entry, maintenance and frequency of Ca^{2+} oscillations in human carcinoid cancer cell lines and support a role for ORAI 1 in formation of tumors in mouse liver

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