Interaction of casein phosphopeptides with channels involved in calcium absorption and modulation of proliferation rate and apoptosis of human intestinal cell lines

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## ABBREVIATIONS

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1,25-Dihydroxycholecalciferol</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CPPs</td>
<td>Casein phosphopeptides</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Free calcium ions</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;o&lt;/sub&gt;</td>
<td>Extracellular calcium concentration</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium Sensing Receptor</td>
</tr>
<tr>
<td>DiBAC&lt;sub&gt;4&lt;/sub&gt;(3)</td>
<td>Bis-(1,3-dibutylbarbituric acid) trimethine oxonol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(2-aminoethyleter)N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>Fura-2/AM</td>
<td>Fura-2 acetoxymethyl ester</td>
</tr>
<tr>
<td>Fura-2</td>
<td>Fura-2</td>
</tr>
<tr>
<td>KRH SD</td>
<td>Krebs Ringer Hepes buffer</td>
</tr>
<tr>
<td>KRH MOD</td>
<td>Krebs Ringer Hepes buffer modified</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>TRPV6</td>
<td>Transient receptor potential cation channel subfamily V member 6</td>
</tr>
<tr>
<td>VDR</td>
<td>Nuclear vitamin D receptor</td>
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1. State of the art
CALCIUM HOMEOSTASIS IN THE INTESTINAL CELLS

Calcium is an essential micronutrient in the human diet and is derived mainly from milk and dairy products (78%), while fewer derived from fruits and vegetables (20%), and even fewer from meat and fish (5%). The absorption of dietary calcium is intrinsically related to the chemical nature of the food source and to the individual nutritional, metabolic and physiological status. Even the compounds introduced in combination with calcium may affect its absorption, such as phytate, oxalate, phenolic compounds, lactose, phosphorus, and of course vitamin D, which plays a decisive role in this process. The phytate in cereals and legumes, for example, form insoluble or poorly soluble complexes with calcium in alkaline pH, making calcium ions unavailable for absorption (Kitts and Yuan 1992), and some pectins drastically inhibit the intestinal absorption of calcium and other minerals (Gueguen and Pointillart 2000). On the other hand several species of fibers (cellulose, hemicellulose, lignin and non-cellulosic polysaccharides) do not seem to directly influence the absorption of the ion, but different non-digestible carbohydrates and oligosaccharides are able to increase its absorption from the distal part of intestines, by increasing the bacterial fermentation and lowering the pH of intestinal environment (Gueguen and Pointillart 2000). Approximately 99% of calcium in the body is in the form of calcium phosphate, the main constituent of bone mineral (Bronner 1988). It follows that the bulk of the ingested calcium is accumulated and retained in the skeletal tissue. The rate of deposition of calcium in bones is high in the newborn and decreases until the end of the development of individual, in parallel, the rate of removal of calcium from the bones tends to equalize the deposition rate (Bronner and Pansu 1999). The process of deposition and removal of calcium from bone is under the control of hormones such as parathyroid hormone and calcitonin, which along with vitamin D regulate the homeostasis of calcium ions in the body, also intervening in the kidneys and intestines. It is widely accepted there are two pathways of absorption: one transcellular, the other paracellular. Transcellular Ca^{2+} absorption is secondary active absorption, requiring ATP hydrolysis by the Ca^{2+} dependent ATPase in the basolateral membrane; it is mediated, can occur at luminal Ca^{2+} concentrations below those in plasma, and is stimulated by 1,25(OH)_{2}D_{3}. In contrast, paracellular absorption is passive, being driven by high Ca^{2+} concentrations through tight junctions down transepithelial electrochemical gradients; it is often described as vitamin D independent. In these conventional terms, active, transcellular absorption is a saturable process occurring at luminal concentrations up to approximately 5 mM, whereas paracellular absorption displays linear or diffusive kinetics between 5 and 200 mM Ca^{2+}. 
The relative contribution of transcellular and paracellular components to total Ca\(^{2+}\) absorption is a matter of some debate but principally depends on the amount and concentration of ionized Ca\(^{2+}\). A current, general view is that the transcellular component accounts for approximately 50% in duodenum, decreasing to 20% in jejunum and 0% in ileum. However, because the transcellular component depends on the Ca\(^{2+}\) content of the diet, such figures are merely guidelines. Thus, at low Ca\(^{2+}\) intakes, when the transcellular component is upregulated by 1,25(OH)\(_2\)D\(_3\), paracellular flow accounts for perhaps <10% of duodenal absorption. In contrast, at high intakes, transcellular absorption is downregulated, so that paracellular flow then accounts for >90% of duodenal absorption. Since the absorptive capacity of the duodenum is diminished at high intakes, the majority of Ca\(^{2+}\) absorption is said to occur by paracellular flow at high concentrations in the jejunum and ileum (Kellett 2011).

There are two main models of active, transcellular intestinal Ca\(^{2+}\) absorption, regulated by genomic or non-genomic pathways (Figure 1). The most widely accepted is the facilitated diffusion model; it comprises three steps, involving TRPV6 (CaT1), a member of the transient receptor potential vanilloid subfamily, calbindin-D\(_{9K}\), and PMCA1b, the predominant form in intestine (Choi and Jeung 2008; Hoenderop, et al. 2005; Peng, et al. 2003). In the duodenum, apical Ca\(^{2+}\) entry into the enterocytes occurs through TRPV6, which is activated by hyperpolarization and is relatively insensitive to L-type channel inhibitors (Peng, et al. 1999; Suzuki, et al. 2008). Transport across the apical membrane is strongly favored by an electrochemical gradient. Cytosolic diffusion of Ca\(^{2+}\) is facilitated 70-fold by binding to calbindin-D\(_{9K}\), which acts as a Ca\(^{2+}\) ferry or fire-bucket brigade. Finally, Ca\(^{2+}\) is transported across the basolateral membrane against the electrochemical gradient via PMCA1b. About 80% of Ca\(^{2+}\) exits via this route, the remaining 20% via the Na\(^+\)/Ca\(^{2+}\) exchanger.

Components of the facilitated diffusion model are strongly upregulated by 1,25(OH)\(_2\)D\(_3\) through the vitamin D receptor (VDR) or by a low-Ca\(^{2+}\) diet. Conversely, both are downregulated by high dietary Ca\(^{2+}\) intake and low 1,25(OH)\(_2\)D\(_3\). PMCA1b is also regulated by 1,25(OH)\(_2\)D\(_3\) (Johnson and Kumar 1994). The protein and mRNA expression levels of TRPV6, calbindin-D\(_{9K}\), and PMCA1b, as determined by immunocytochemistry and quantitative polymerase chain reaction, are highest in duodenum and decrease distally; they are also present in cecum and colon (Armbrecht, et al. 2003; Hoenderop, et al. 2000; Zhuang, et al. 2002). The vesicular transport model for rapid non-genomic regulation is based on work in chick duodenum, which expresses calbindin-D\(_{28K}\) (Khanal and Nemere 2008b; Larsson, et al. 1998). Stimulation of Ca\(^{2+}\) entry by vitamin D acting through the 1,25(OH)\(_2\)D\(_3\)-MARRS
(membrane-associated, rapid response steroid-binding) receptor increases Ca\(^{2+}\) in the subapical region within minutes, disrupting actin filaments (Nemere 2005).

The ensuing endocytotic vesicles are transported by microtubules to fuse with lysosomes and, ultimately, with the basolateral membrane to secrete Ca\(^{2+}\) and calbindin-D\(_{28K}\) into the circulation (Figure 1). Lysosomal/basolateral fusion may involve control of local Ca\(^{2+}\) concentrations by influx through basolateral L-type channels in conjunction with efflux through PMCA1b, consistent with the observation of rapid Ca\(^{2+}\) recycling across the basolateral membrane (Khanal and Nemere 2008a).

Figure 1. Calcium transport in rat jejunum. A central feature is that transcellular absorption is regulated by alternative depolarization and repolarization of the apical membrane during and between digestive periods, respectively. There are two active transcellular pathways for Ca\(^{2+}\) absorption, which have complementary roles: entry to one is mediated by TRPV6, which is activated by hyperpolarization of the apical membrane, and entry to the other is mediated by Cav1.3, which is activated by depolarization. Entry through TRPV6 controls three potential routes: 1) the facilitated diffusion pathway by calbindin-D\(_{9K}\); 2) simple diffusion of free Ca\(^{2+}\); and 3) the vesicular transport pathway, in which Ca\(^{2+}\) and calbindin-D\(_{9K}\) are simultaneously released into the plasma by vesicle fusion with the basolateral membrane. TRPV6, calbindin-D\(_{9K}\), and PMCA1b are also upregulated genomically by vitamin D. Cav1.3 is activated by Na\(^+\)/glucose co-transport through SGLT1 and by other depolarizing nutrients, including amino acids and oligopeptides. The transcellular pathway is not yet established but is independent of calbindin-D\(_{9K}\) and, probably, vitamin D. Absorption through Cav1.3 also regulates apical GLUT2 insertion. Paracellular absorption is shown as a minor component, given that Cav1.3, alone or in conjunction with TRPV6, offers alternative explanations for some phenomena currently attributed to paracellular flow. Dashed lines: with arrowhead, activation; with bar, inhibition (Kellett 2011).
A fundamental issue is how intestinal Ca\textsuperscript{2+} absorption occurs under the depolarizing conditions of digestion. The products of the digestion of disaccharides, peptides, and amino acids are strongly depolarizing and are generated throughout jejunum and ileum, which are the absorptive regions with the longest transit time for food. Two possible mechanisms might operate under depolarizing conditions: nutrient-induced paracellular flow and/or activation of L-type channels. It is widely accepted that the major pathway of Ca\textsuperscript{2+} absorption attributed to paracellular flow occurs in jejunum and ileum, which have a much more depolarizing environment than duodenum after a meal.

Nevertheless, it is also widely accepted that intestinal epithelial cells do not contain L-type channels, since enterocytes are activated by hyperpolarization (Petersen and Fedirko 2001). This view was reinforced by the discovery of ECaCs, which do not contain the S4 voltage sensor present in L-type channels and are activated by hyperpolarization in the range of -60 to -150 mV (Figure 2). Hence, TRPV6 was found to be located largely in the duodenum, which has a relatively polarizing environment and a very short chyme transit time. The most widely accepted mechanism of transcellular Ca\textsuperscript{2+} absorption, therefore, does not, indeed cannot, explain the fundamental issue of absorption during digestion.

**Figure 2.** Structural organization of TRPV6. A: the epithelial Ca\textsuperscript{2+} channels are 730 amino acids long with a predicted molecular mass of 83 kDa. TRPV6 contain a core domain consisting of 6 transmembrane (TM) segments. In addition, a large cytosolic amino and carboxy terminus are present containing ankyrin repeats. Between TM5 and TM6 there is a short hydrophobic stretch predicted to be the pore-forming region of these channels. Inner and outer side of the membrane is indicated. B: potential regulatory sites in the amino and carboxy tail of TRPV6 including ankyrin repeats and PDZ motifs and conserved PKC phosphorylation sites (Hoenderop et al. 2005).
There is, in fact, clear evidence that L-type channels are present in enterocytes. Of particular relevance is the demonstration of L-type channels in the apical membrane of Atlantic cod intestine (Larsson et al. 1998). At 10 mM Ca\(^{2+}\), imaging experiments revealed that K\(^+-\)induced depolarization of Fura-2 loaded isolated cells resulted in a wave of Ca\(^{2+}\) entering at the apical membrane and exiting at the basolateral membrane about 1 minute later (Larsson et al. 1998). The associated increase in intracellular Ca\(^{2+}\) levels was mimicked by Bay K 8644 and blocked by nifedipine (5 mM).

A role for apical Ca\(^{2+}\) entry was confirmed by single-pass perfusion of rat jejunum in vivo; entry could not involve TRPV6, which lacks the S4 voltage sensor and is opened by hyperpolarization, i.e., between -60 and -150 mV (Hoenderop, et al. 1999; Peng et al. 1999). Furthermore, TRPV6 is located predominantly in duodenum, (Hoenderop et al. 2000; Zhuang et al. 2002) which has a relatively polarizing environment, modest glucose absorption, and negligible apical GLUT2 insertion.

The depolarizing conditions in jejunum pointed to an apical L-type channel. Transcripts of the non-classical neuroendocrine Cav1.3 α1 pore-forming subunit and an associated β3 subunit were amplified from jejunal mucosal cDNA using an homology-based polymerase chain reaction approach (Morgan, et al. 2007); no other L-type channel was detected. Protein expression of both subunits was localized by Western blotting and immunocytochemistry to the apical membrane. Ca\(^{2+}\) absorption displayed L-type characteristics (Morgan et al. 2007; Morgan, et al. 2003).

Cav1.3 is a second channel capable of active, transcellular Ca\(^{2+}\) absorption in intestine. Cav1.3 is activated by membrane depolarization. Cav1.3 can therefore operate under conditions of sustained, weak membrane depolarization at low-voltage thresholds, such as those generated by nutrient absorption around -47 mV.

TRPV6 is strongly activated at \(V_m\) values from -60 mV to -150 mV and Cav1.3 at more positive potentials from +10 mV to -20 mV. This suggests TRPV6 and Cav1.3 have complementary roles in Ca\(^{2+}\) entry. The scenario below assumes that TRPV6 and Cav1.3 make independent contributions to absorption (Nakkrasae, et al. 2010).

In terms of extending the current perspective of Ca\(^{2+}\) absorption, the simplest proposal is that rapid changes in Ca\(^{2+}\) absorption during digestion occur as a consequence of alternate depolarization and repolarization during and between digestive periods; the changes are achieved by alteration in the contributions of Cav1.3 and TRPV6, respectively, in response to changes in \(V_m\). In this view, Cav1.3 plays the dominant role during digestion, especially when diet and Ca\(^{2+}\) are plentiful, so that TRPV6 activity is downregulated genomically and inhibited by changes in \(V_m\) when glucose concentrations at the apical membrane can reach 30–100 mM (Mace, et al. 2007).
depolarizing digestion products, such as amino acids and peptides, may activate Cav1.3 and inhibit TRPV6.

Moreover, depolarization is augmented by increased synthesis of disaccharidases and nutrient transporters. On the other hand, TRPV6 plays a dominant role as a powerful scavenger under the polarizing conditions between meals.

In a situation in which luminal Ca$^{2+}$ is less than plasma Ca$^{2+}$ it is essential that TRPV6 be both activated by apical membrane repolarization and up-regulated by vitamin D to prevent massive loss of Ca$^{2+}$ from the body. Perhaps this is the fundamental reason why vitamin D and TRPV6 are so important in Ca$^{2+}$ homeostasis overall. The interplay of TRPV6 and Cav1.3 is essential to maintain the exceptionally tight control of free Ca$^{2+}$ concentration in the extracellular space over the entire range of dietary intake, both of Ca$^{2+}$ and other nutrients (Kellett 2011).

Cav1.3 mediates entry to a second active transcellular pathway in the heart of the digestive center, regulated by depolarizing nutrients and hormones. In particular, rapid glucose- and prolactin-induced Ca$^{2+}$ absorptions through Cav1.3 are both independent of calbindin-D$_{9k}$ and probably of vitamin D. TRPV6 is activated by hyperpolarization; hence, Cav1.3 does what TRPV6 cannot, i.e., operate optimally during dietary Ca$^{2+}$ intake under the depolarizing conditions of digestion.

A novel, integrated view of transcellular Ca$^{2+}$ absorption is presented based on the idea that Cav1.3 and TRPV6 have independent and complementary actions, being activated by depolarization and repolarization during and between digestive periods, respectively (Kellett 2011).
REGULATORY MECHANISMS OF CALCIUM INSIDE THE CELL

Calcium, in addition to playing a nutritional role, is also an important second messenger and a regulator of cellular functions. It is involved in many cellular responses, such as muscle contraction, nerve transmission, secretion, also plays an important role in regulating cell growth and differentiation, and apoptosis.

The concentration of intracellular calcium, [Ca\(^{2+}\)]\(_i\) (0.1-0.2 µM) is four orders of size lower than extracellular calcium, [Ca\(^{2+}\)]\(_o\) or (1-2 mM), thus the maintenance of this gradient, and the rapid increase in [Ca\(^{2+}\)]\(_i\) (10-20 times in a few milliseconds), observed after stimulation with an agonist, are the result of coordinated action of several mechanisms that include: influx and efflux of calcium through the cytoplasmic membrane, intracellular calcium stores and buffer systems (Figure 3).

The entry of calcium from the extracellular environment occurs through channels present at the plasma membrane; according to the mechanism of activation is possible to determine the different types (Kitts and Yuan 1992; Nelson and Cox 2006) Channels activated by the presence of an extracellular chemical signal (ligand), which, according to their mechanism of action can be further subdivided into:

- **ROCCs** (Receptor-Operated Ca\(^{2+}\) Channels): calcium channel regulated by a receptor where the ligand binding site and the channel are included in the same protein or part of the same complex. These channels open as a result of competitive binding to the receptor without the involvement of diffusible second messengers.
- **GOCCs** (G protein-Operated Ca\(^{2+}\) Channels), channels directly coupled to receptors through G protein.
- **SOCCs** (Stores-Operated Ca\(^{2+}\) Channels), channels activated by depletion of intracellular stores, with a mechanism of activation still uncertain.
- **SMOCCs** (Second Messenger-Operated Ca\(^{2+}\) Channels): channels activated by a second messenger. The opening of these channels is controlled by a diffusible cytosolic messenger whose production is due to ligand-receptor interaction.

Voltage-activated channels (VOCCs), whose presence was detected in both excitable cells, such as nerve and muscle fibers and in no-excitatory cells, such as fibroblasts. The opening of these channels occurs as a result of depolarization of the plasma membrane. The VOCCs can be divided into different classes according to electrophysiological characteristics and sensitivity to different inhibitors, making the complexity and functional characteristics of these channels in different types of cells.

In the cell there are several ways of buffering or sequester calcium to prevent an uncontrolled increase in [Ca\(^{2+}\)]\(_i\) prolonged.
The transport processes located in the plasma membrane are able to guarantee the maintenance of an adequate level of cytosolic free calcium ions and consist of two main systems for extrusion of the ion, ensuring the maintenance of the concentration gradient: the Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchanger. The Ca\(^{2+}\)-ATPase is an active transport system, ubiquitous in eukaryotic cells, which can bind intracellular calcium with high affinity and release it in the environment outside the plasma membrane by consuming ATP. The Na\(^+\)/Ca\(^{2+}\) exchanger is present in only a few cell types, especially in excitable cells (muscle and nerve), coupling the release of one calcium ion against gradient to the entry of three sodium ions into cells following the gradient (Putney 1999).

There are other mechanisms which are key in maintaining ion homeostasis, such as the binding to cytosolic proteins and the accumulation in intracellular compartments. In the cell, as well as in mitochondria and Golgi apparatus, where calcium concentrations are extremely high, the main organelle involved in storage and in the rapid exchange of calcium after cell stimulation is the endoplasmic reticulum, and, in particular, one specialized sub-compartments: the calciosoma. Studies on skeletal muscle (Lamb and Stephenson 1992) have shown that the accumulation of calcium in the sarco/endoplasmic reticulum occurs against the gradient through the Ca\(^{2+}\)-ATPase, present in the membrane of this organelle, and capable of transporting two calcium ions into the lumen of the reticulum for each molecule of hydrolyzed ATP.

Within the intracellular stores, most of the calcium is not present in ionic form, but is bound to proteins with low affinity (Kd = 1-4 mmol/ L) and high binding capacity (25-50 calcium moles/mole of protein) toward the ion, which enhance the ability to storage of the endoplasmic reticulum (Wasserman and Fullmer 1995): the calsequestrin, present mainly in muscle cells, and calreticulin, common in a greater number of cell types. The release of calcium from intracellular stores is due to inositol 1,4,5 trisphosphate (IP3): it derives from the cleavage of phosphatidyl-inositol-4,5-bisphosphat by the action of phospholipase C, coupled with a G protein. The IP3 acts as a second messenger by binding to its receptor, located at the membrane of the endoplasmic reticulum, activates calcium channel response resulting in the release of the ion in the cytosol (Berman 1999). This process depends critically on the biphasic [Ca\(^{2+}\)]: at low concentrations (<0.3 mM) calcium acts as a co-agonist of IP3 (thus exerting a positive control on his release from the endoplasmic reticulum), whereas at higher concentrations exerts a negative control on the release of Ca\(^{2+}\)-induced by IP3, inhibiting the receptor and thus preventing further release of Ca\(^{2+}\) (Orrenius, et al. 2003).
Inside the eukaryotic cell, there are also systems that are able of buffering large transient increases in $[\text{Ca}^{2+}]_i$ that could lead to the activation of enzymatic reactions harmful for the cell. These are proteins with an heterogeneous affinity and ability to bind $\text{Ca}^{2+}$, which take place inside the cell $\text{Ca}^{2+}$-dependent enzymatic action (for example, phosphorylase) or regulatory (eg calmodulin). This last plays an important role in the modulation of a variety of physiological processes (such as $\text{Ca}^{2+}$-ATPase activity of the membrane), as well as representing the cytoplasmic protein important in binding $\text{Ca}^{2+}$, in virtue of its high concentration (approximately 1% of total cellular proteins) and the presence in it of four binding sites for $\text{Ca}^{2+}$.

**Figure 3.** Intracellular $\text{Ca}^{2+}$ homeostasis. The extracellular $\text{Ca}^{2+}$ can enter the cell through voltage-gated channels permeable to $\text{Ca}^{2+}$ (VOOCs), channels sensitive to the concentration of intracellular $\text{Ca}^{2+}$ (SOOCs), channels through $\text{G}$ protein-coupled receptors (ROCCs). $\text{Ca}^{2+}$ in the endoplasmic reticulum is released into the cytosol through channels opened by IP3. The PLC hydrolyzes PIP2, producing IP3 and DAG. The intracellular concentration of calcium is returned to baseline by extrusion of the ion mediated by $\text{Ca}^{2+}$-ATPase present on the plasma membrane (PMCA) and on the membrane of the endoplasmic reticulum (SERCA), or by the antiport $\text{Na}^+/\text{Ca}^{2+}$ (Orrenius et al. 2003).
MORPHOLOGY OF THE INTESTINAL MUCOSA

After birth, the intestinal mucosa is constantly subjected to stimuli from the external environment and plays a key role in absorption and metabolism of nutrients, drugs and other substances in transit through the intestinal lumen. Intestine enterocytes are alternating with goblet cells and are based on a layer of connective tissue, the lamina propria, thus going to constitute the intestinal mucosa. To increase the total absorption area, the mucosa rises in the finger-like formations, the intestinal villi, in which enterocytes are arranged in a very specific way (Dalle Donne, et al. 2010).

At the base of the villi, in the formations known as crypts, the cells are functionally "stem cells", which are characterized by high self-replicating capacity, which leads them to be a clone of cells designed to maintain this proliferative rate. In addition there are cells moving away from the crypt reaching the surface of the villi. During this route the cells change, no longer proliferate and assume characteristics of differentiated cells (de Santa Barbara, et al. 2003) (Figure 4). Differentiated epithelial cells have a well developed junctional apparatus, which divides the plasma membrane in a basolateral and apical compartment, causing a functional polarity. The junctions most representative in the epithelial cells are adherent junctions that, in addition to promote adherence and maintain tissue integrity, facilitate intercellular communication (Dejana 2004). Among the markers of epithelial differentiation, a major role is played by the E-cadherin, a transmembrane protein present in adherent junctions, which has an extracellular portion, a transmembrane domain and cytoplasmic tail. The extracellular domain of E-cadherin establishes homophilic calcium-dependent interactions with similar molecules of adjacent enterocytes, while its intracellular tail binds to proteins such as cytoplasmic β-catenin, which are joined to the cytoskeletal actin filaments (Farquhar and Palade 1963). This anchoring allows both the cytoskeleton stabilization and the dynamic adjustment of the opening and closing of joints themselves (Dejana 2004). At the apex of the villus cells, now have reached the final stage differentiation and viability, undergo programmed cell death, apoptosis. The balance between the high proliferative rate in the crypts and apoptosis at the apex of the villi allows to avoid the production of a mass of cells along the axis of the villi, which lead to the formation of microadenomas, polyps and carcinomas.
Figure 4. Morphological representation of the organization of the small intestine ("Fondamenti di nutrizione umana"; A.M. Costantini, C. Cannella, G.Tomassi; Il Pensiero Scientifico Editore).
CALCIUM AND CARCINOGENESIS

Colorectal tumors are one of the most common forms of cancer (Jemal, et al. 2005) and are the second leading cause of cancer mortality in industrialized societies (Greenwald 1992). A common feature in the development of this disease is an increased proliferative activity of intestinal cells together with the arrest of cell growth control (Brown and Attardi 2005) and the loss of a defined morphology (Christiansen and Rajasekaran 2006).

Recent epidemiological studies have shown that the incidence of colon cancers is inversely proportional to intake of dietary calcium and to the presence of vitamin D in our body in its active form, 1,25(OH)₂D₃ (Jacobs, et al. 2009; Peterlik, et al. 2009).

At the level of colon cells, the concentration of calcium needed to maintain a balanced proliferative rate is significantly lower than required by cells such as fibroblasts and hepatocytes (1.0-1.5 mm versus 0.8-2.2 mM) (Whitfield, et al. 1995). An excess of extracellular calcium, such as that due to a meal, which can reach values of 6-8 mM (Bronner and Pansu 1999), has as a direct consequence the blockade of proliferation and the expression of a differentiated phenotype, characterized by the presence of adherent junctions between cells (Whitfield 2009).

The molecular mechanisms underlying this transformation see the involvement of two main mechanisms: the Wnt mechanism and CaSR (Calcium Sensing Receptor). The Wnt mechanism, consisting of a series of glycoproteins released by cells of mesenchymal stromal crypts, controls cell proliferation through the complex between the nuclear β-catenin and the transcription factor 4 (TCF4), a nuclear transcription factor that belongs to the family of T cell receptors (Korinek, et al. 1997). This complex activates a number of genes required for cell proliferation, including c-myc, and cyclinD1, leading to the expression of an anti-apoptotic protein (survivin) and to the inhibition of the "cycling p21 cell-stopper", thus allowing cells at the base of the crypts to replicate (Chakrabarty, et al. 2005).

The increase in extracellular calcium concentration activates the entry of these ions into the cells through specific channels, including the TRPV6 (Transient Receptor Potential vanilloid Calcium channel, even channel CAT1), belonging to the superfamily of channels Transient Receptor Potential (TRP) (Peng et al. 1999) and activates the expression of the CaSR. The CaSR, a G protein-coupled receptor having as the main physiological ligand calcium, is able to "detect" even minimal variations in the extracellular concentration of this ion and to "translate" into mechanisms of activation/inhibition of cellular functions (Gama, et al. 1997). Physiologically, is expressed at the apex of the villi during the differentiation of enterocytes, which are in contact with a calcium concentration significantly higher than that found at the bottom.
of the crypts (Brenner, et al. 1998; Chakrabarty et al. 2005). CaSR invokes calcium ions into cells, thus establishing a cycle calcium-CaSR that has as its ultimate effect the coordination of the events of proliferation-differentiation-apoptosis. One of the first events of the calcium-CaSR cycle is the translocation of β-catenin from the nucleus to the cytoplasm, with dissociation of the complex TCF4-β-catenin and the appearance of the APC (adenomatous polyposis coli protein) in the cytoplasm. These events induce the expression of E-cadherin (Chakrabarty et al. 2005) which promotes the structural organization of adherent junctions between adjacent epithelial cells. At this stage, the cells acquired a differentiated phenotype, associated with the inhibition of the survivin protein, and the further migration at the apex of villus, leads to complete their life cycle with apoptosis. All these steps are tightly regulated in healthy cells at the genomic and post-genomic level (Clevers 2006; Hoffman, et al. 2004; Pinto and Clevers 2005) (Figure 5).

Vitamin D can modulate gene expression in ways specific to each tissue, primarily by inhibiting cell proliferation, inducing cell differentiation and apoptosis, processes which, in turn, may protect cells from tumor transformation (Chiang and Chen 2009). The chemopreventive action of vitamin D, similar to calcium, is expressed through the interaction with calcium both activating/inhibiting antiproliferative/pro-differentiating ways and promoting the expression of CaSR (Peterlik et al. 2009).

Enterocytes that undergo a premalignant transformation lose the ability to respond to the differentiating stimulus represented by a high concentration of extracellular calcium. In addition, clones of cells appears quite unable to express the CaSR, resistant to apoptosis and thus active proliferating, characterized by the presence of nuclear β-catenin (Chakrabarty, et al. 2003; Chakrabarty et al. 2005).

In colon adenocarcinoma cells the increased extracellular calcium concentration, due to the diet, results in the stimulation of proliferation, while the same concentration of extracellular calcium has a proapoptotic effect on the neighboring healthy cells. Therefore the increase of calcium in the diet has a chemopreventive effect, as suggested by several studies in healthy intestinal cells, whereas in cells of adenomatous crypts have the opposite effect of being a promoter of carcinogenesis (Bostick, et al. 1993; Kleibeuker, et al. 1993).
Figure 5. Model for the role of β-Catenin/TCF in the early stages of intestinal tumorigenesis. Modified by van de Wetering et al., Cell (2002).
TUMOR CELL LINES AS MODELS OF INTESTINAL EPITHELIUM

It is not possible to stabilize in vitro culture cells from biopsies of normal human intestine, adult or fetal, showing the structural and functional properties of intestinal epithelial physiology, so human tumor cell lines are commercially available and commonly used as models of intestinal epithelium.

The established cell lines from explants of human colon carcinomas have represented and still represent an important advantage both in experimental, to understand the mechanisms intervening in the development of malignant disease of that tissue, and for the study of the major biological processes that occur in the intestinal mucosa. Tumors of the colon, unlike those of the small intestine, are quite common and have provided considerable theoretical and practical knowledge useful for the preparation of stable cell lines used as in vitro model of this tissue. Among these, the HT-29 and Caco2 cell lines represent the most studied and used experimentally as they have the ability, if properly differentiated, to display the typical characteristics of a mature enterocytic phenotype (Quaroni and Hochman 1996).

**HT-29 cell line**

This line was established in culture from a human colon adenocarcinoma in 1964 by Jørgen Fogh.

The HT-29 cells when appropriately differentiated provide a good and faithful model of polarized intestinal epithelium, which has the function to generate concentration gradients of nutrients and electrolytes between the interior and exterior of the cell. This polarity reflects the morphology and protein composition of two different asymmetric surfaces:

- a baso-lateral portion, characterized by the presence of protein complexes suitable for carrying substances, such as the electrogenic pump Na⁺-K⁺/ATPase. The cells of this region express antigens of the major histocompatibility complex (MHC class I and II); synthesize receptors for dimeric IgA; express functional receptors for peptide and hormones.

- an apical portion which presents the typical structure of the brush border associated with the microvilli, whose membrane secretes several hydrolytic enzymes such as disaccharidases, alkaline phosphatase, peptidase, all involved in terminal digestion of carbohydrates in the diet. Is also able to synthesize molecules with transport function for amino acids and sugars.

The differentiated HT-29 cells are considered to be a heterogeneous cell population comprised by mucus-secreting cells, similar to goblet cells, and by entero-endocrine cells. At morphological level, as well as brush border, also develop a typical junctional
apparatus, consisting of tight junctions, adherent junctions and desmosoms, indicating a high degree of differentiation. All these morphological and functional characteristics become evident when the cell line HT-29 is induced, in culture, to differentiate. Normally these cells grown in vitro under standard conditions do not show features of enterocytes, but the differentiation process can be induced by manipulating the growth conditions, for example: i) by replacing glucose with galactose (Pinto M 1983), ii) by subcultures in the Roswell Park Memorial Institute (RPMI) medium at low glucose (2 g/L)(Cosentino, et al. 2010, Polak-Charcon, et al. 1989; Zweibaum, et al. 1985). For these features, the HT-29 cells are considered a pluripotent enterocitic cell line, able to express significant degrees of differentiation depending on culture conditions to which cells are subjected (Zweibaum A 1991).
The HT-29 cells grown in DMEM, high glucose medium (4.5g/L) show an undifferentiated phenotype. They form a multistratified epithelium of unpolarized cells, that have in the apical portion poorly developed microvilli and no junctional apparatus (Cosentino et al. 2010; Polak-Charcon et al. 1989). The enzyme activity, although at a limited extent, is still present (Pinto M 1983; Zweibaum A 1991).Within the parental population, when maintained in this standard culture conditions, there is a small percentage of cells (about 4%) that spontaneously undergoes to differentiation along both the enterocytic and mucous secreting goblet lineages (Lesuffleur, et al. 1990). These observations confirms the pluripotentiality of this cell line (Huet, et al. 1987).
Four different cytotypes reside within the mucosa of the intestine: the enterocyte, the goblet mucous secreting cell, the enteroendocrine cell and the Paneth's cell (Huet et al. 1987).

**Caco2 cell line**
The Caco2 cells have been demonstrated to be particularly useful as enterocytic model. This line represents a model with notably growth-related differentiation potential along the enterocytic lineage since it is able to spontaneously reach an elevated degree of maturity when maintained in a post-confluence status, in standard culture conditions (Pinto M 1983; Simon-Assmann, et al. 2007), although this methodology does not fully reflect the characteristics of physiological epithelium, subjected by a fast cell turn over.
Like the HT-29 cells, this cell line derived from a human colon adenocarcinoma. A limiting factor of the maintenance of culture at post-confluence is not fully reflect the characteristics of physiological epithelium.
The enterocytic differentiation is determined by a series of steps with precise chronological, morphological and functional characteristics. Moreover this process is
strikingly related to the cell proliferation, since it takes place at the post-confluent status, a condition in which the proliferation gradually decreases. Ferraretto and colleagues established a new original methodological approach to induce a differentiated phenotype in these cells. The cell growth conditions were carefully standardized so as to provide good reproducibility and a routine procedure that allowed a high degree of cell differentiation to be reached without reaching the long-term post-confluence status (Ferraretto, et al. 2007). When cells reached 100% confluence, as checked by phase-contrast microscopy (Olympus, IX50, Tokyo, Japan), they were trypsinized after one day post-confluence (Trypsin-EDTA 0.05%-0.02%), diluted (usually 1:3) and then seeded again in a new plastic flask. Each trypsinization and successive dilution in a new flask was considered as a cell passage.

The differentiated Caco2 cell shows the typical enterocytic morphology with a columnar shape as well as the brush border apparatus with its characteristic glycocalyx. The apical microvilli develop in a well-organized structures homogeneously distributed on the absorptive surface. In a terminally differentiated culture it is possible to observe the dome structures as well as, at the ultrastructural level, the complete junctional apparatus, indexes of the formation of a polarized epithelium in which the transepithelial transport mechanisms, for ion, water and micronutrients, are activated (Ferraretto et al. 2007; Simon-Assmann et al. 2007). Functionally the trans-epithelial electrical resistance (TEER) and permeability of marker molecules could be used to monitor the integrity of the cell layer (Sambuy, et al. 2005). This cell line, in particular, is the model most used in studies of transport of xenobiotics and nutrients due to its ability to differentiate into absorbent epithelium. Although derived from an adult human colon (where microvillar hydrolases are not present), the distinctive characteristic of the Caco2 cells is the ability to synthesize and to release a panel of hydrolytic enzymes (sucrase-isomaltase, lactase, aminopeptidases and alkaline phosphatase) as marker of enterocytic differentiation typical of the small intestinal villus cells (Pinto M 1983; Quaroni and Hochman 1996; Zweibaum A 1991). The enzymatic activities are strikingly growth-dependent: different hydrolases are expressed and activated at different times along the differentiation process and they increase along the post-confluence maintenance (Zweibaum A 1991).
CASEIN PHOSPHOPEPTIDES (CPPs):
BIOACTIVE PEPTIDES DERIVED FROM CASEIN

About 80% of the bovine milk proteins is represented by the caseins, a family of phosphoproteins (αs1-, αs2-, β- and κ-caseins) synthesized by the mammary glandular epithelium. In their native state they do not explicate any known biological activity, but they constitute a good nutritional source of amino acids (Schlimme and Meisel 1995).

Caseins contain a series of peptides, inactive when hidden within the precursor protein, that once released following the gastrointestinal digestive enzymatic processes, are able to demonstrate a biological activity (Meisel and Frister 1988; Naito, et al. 1972). Among these peptides it is interestingly to point out the opioid receptor agonist (casomorphines) and antagonist (cosoxines) peptides, anti-hypertensive (casochinines) and anti-thrombotic (casoplatelines) active peptides and immunostimulating peptides (immunopeptides) (Meisel and Schlimme 1990; Shah 2000).

In addition, among the bioactive peptides, there are also the casein phosphopeptides (CPPs), αs1-, αs2-, β-caseins derived phosphorilated peptides, able to bound and solubilize minerals, such as calcium, by its serine-associated phosphate groups, preventing their precipitation in the calcium-phosphate salt form. This action is responsible for the increased bioavailable calcium fraction in the small intestine, and particularly at the ileum level (Berrocal, et al. 1989; Erba, et al. 2002; Meisel and Olieman 1998). Indeed, the dephosphorilated peptides are not able to bind calcium, while the casein phosphorilation increases both its binding capacity and the resistance to proteolysis (FitzGerald 1998).

The high calcium bioavailability of milk and dairy products has been attributed to the CPPs formation, along the digestive tract; moreover the high number of phosphorilated serine residues, -Ser(P)-, and the consequent high negative charge density, account for the resistance of CPPs to further proteolytic digestion and, thus, for their accumulation in the distal small intestine, e.g. the ileum (McDonagh and FitzGerald 1998).

Despite of the differences in length and aminoacidic composition, all the CPPs share a highly polar “acidic domain” containing three serine phosphate cluster and glutamyl residues: -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-. This sequence is highly conserved between the species and represent the binding site for mono and divalent ions, and among them the calcium (Kasai, et al. 1992; Schlimme and Meisel 1995; West and Towers 1976).
The production of CPPs can be carried out in vitro starting from the bovine sodium caseinate, through digestion by pancreatic enzymatic preparations presenting the trypsic as the main activity (Sato, et al. 1991).

Their binding ability varies from 0.4 to 0.6 mg of Ca\(^{2+}\)/mg of CPPs, whereas the ion solubilizing ability varies from 7.4 to 24 mg of Ca\(^{2+}\)/mg of CPPs. There is a net distinction between the calcium binding ability and the ability to solubilize it; moreover depending on the enzymatic preparation utilized, the calcium solubilizing ability changes, probably on the base of the upstream and downstream aminoacidic sequence (McDonagh and FitzGerald 1998).

The calcium binding affinity constant, as determined by capillary electrophoresis, is comprised between 10\(^{-2}\) -10\(^{-3}\) M\(^{-1}\) (Meisel and Schlimme 1990); this low affinity could facilitate the ion release during the intestinal absorption phenomena.

In the last few years the studies concerning the physico-chemical CPP structure and the protein structure-calcium binding correlation have been focused on the two CPPs that form in highest amount during the intestinal enzymatic digestion: β-CN(1-25)4P and αs1-CN(59-75)5P. The primary sequences of these two peptides are reported in Figure 6.

![Figure 6](image)

**Figure 6.** Primary structures of the αs1-CN (59-79)5P and β-CN(1-25)4P CPPs purified from bovine caseins. The “acidic motif” characteristic of all CPPs is indicated in bold characters. S means the phosphorylated serine.
BIOLOGICAL ACTIVITY OF CPPs

The first indications about a potential physiological role for CPPs came from the 1950, when Mellander demonstrated that the tryptic digestion-derived casein phosphopeptides were able to increase the bone calcification level in rachitic children without a contemporary vitamin D administration (Mellander and Olsson 1956). Subsequently it was demonstrated that their administration induced an increased calcium absorption in rats (Patrick and Bacon 1957), and their presence in the diet of ovariectomized female rats was able to reduce the bone mineral density loss (Tsuchita, et al. 1996).

The proved ability of CPPs to prevent the precipitation of calcium in the presence of phosphate has suggested the possibility that CPPs could enhance the amount of soluble calcium in the intestinal lumen, thereby increasing the mineral availability for absorption in the small intestine (Kitts and Yuan 1992). Nevertheless, experiments in vitro with intestinal preparations and animals (pigs and rats) fed a soy protein rich diet implemented with CPPs, however, have provided conflicting results, and especially they do not have clarified in any way the molecular mechanism used by these peptides to increase absorption of calcium (Pointillart and Gueguen 1989; Sato, et al. 1986).

On the contrary, in vitro cell studies demonstrated with no doubts that CPPs can affect diverse biological function. The first direct evidence of an interaction between intestinal cells and CPPs came from the observation that in presence of CPPs and extracellular calcium, HT-29 and Caco2 cells, two known human intestinal in vitro cell models, are induced to uptake calcium ions from the extracellular milieu (Ferraretto, et al. 2001). This activity was subsequently recognized to be due to the presence of a structural CPP conformation, conferred by both the phosphorylated ‘acidic motif’ and the preceding N-terminal portion, necessary for the interaction with the cell plasma membrane (Ferraretto, et al. 2003). Instrumental to this CPP aptitude is the formation of stable aggregates with calcium ions (Ferraretto et al. 2003), in fact the cellular response, i.e, the increased intracellular calcium concentration, follows a dose-response relationship similar to that reported for the formation of aggregates, with a maximum at 4 mM extracellular calcium concentration and a CPP dose of 1280 µM, if a purified commercial mixture, or 200 µM, if a single purified CPP (Gravaghi, et al. 2007).

More recent studies have also provided results concerning differences between the intestinal cell lines used, HT-29 and Caco2, in their response to CPPs, evidencing that, regardless of the slight differences (mostly at the quantitative levels) between the two cell lines, the ability of these cells to take up extracellular calcium under CPP stimulation is a molecular attitude expressed upon differentiation (Cosentino et al. 2010). Another demonstration of the interaction between CPPs and intestinal cells can
be derived by two works (Kawahara, et al. 2004; Kitts and Nakamura 2006) presenting experimental evidences that a particular CPP sequence, commercially available as CPP-III, (Meiji Seika, Tokyo, Japan), consisting of about 90% casein phosphopeptides such as bovine αs2-casein (1-32) and β-casein (1-28) and enriched in calcium, modulates the intestinal immune system by enhancing the mRNA expression of TNF-α as well as IL-6 (a cytokine with functions of modulator of cellular differentiation, proliferation and apoptosis) in a dose dependent manner.

The use of primary human osteoblast-like cells, established in culture from trabecular bone samples obtained from waste materials during orthopaedic surgery, has revealed interesting and new potentialities for CPPs when administered. Analogously to what monitored in intestinal in vitro cell lines, CPPs stimulate calcium uptake by primary human osteoblast-like cells and induce an increase in the expression and activity of alkaline phosphatase (ALP), a marker of human osteoblast differentiation. Moreover, the higher fraction of available calcium due to CPPs enhance calcification nodule formation by human SaOS-2 in vitro cell culture (Donida, et al. 2009).
1.1 REFERENCES


Berman MC 1999 Regulation of Ca2+ transport by sarcoplasmic reticulum Ca2+-ATPase at limiting [Ca2+]. *Biochim Biophys Acta* **1418** 48-60.


Patrick H & Bacon JA 1957 The effect of vitamin D upon bone mineralization of Ca45 and Sr89 as chlorides and as phosphopeptides. J Biol Chem 228 569-572.


2. Aim of the study
For several years CPPs were studied in order to prove their usage to increase the fraction of calcium absorbed in the ileum, where they concentrated (FitzGerald 1998), but the difficulties of the both human and animal studies have provided conflicting results (Ferraretto and Fiorilli 2010).

Instead, studies using *in vitro* cell cultures have revealed new possibilities for CPPs, such as the modulation of the intestinal immune system (Otani, et al. 2001) the antiproliferative effects on cultured mammalian intestinal cells (Ganjam, et al. 1997), the modulation of cell viability in human cells cultures (Hartmann, et al. 2000)

In this context, also exist the recent discoveries that CPPs can lead to increases intracellular calcium in human intestinal cancer cell lines HT-29 and Caco2 but only when differentiated (Cosentino et al. 2010) appears interesting for new applications. These results can be considered as well as from the point of view of a resurgence of calcium absorption, also in virtue of the fact that influx of calcium ions induced by certain mitogenic factors may play a role as messengers of cellular events related to the control of proliferation, as well cell viability (Munaron, et al. 2004). In fact, abnormal cell proliferation is often associated with pathological conditions such as inflammation and cancer (Brown and Attardi 2005; Wright, et al. 1994)

It can therefore hypothesize that the ability of CPP to keep calcium ions in a soluble form could be used to abolish the transmembrane calcium gradient, especially in the carcinoma of the colon, where the excess of calcium introduced with the diet favors the hyperproliferation of the cells and the absence of CaSR (Calcium sensing receptor) results in the absence of apoptotic stimuli (Munaron et al. 2004).

In fact, acting on the extracellular calcium concentration it could be possible to modify the proliferative status of tumor cells. If high calcium concentrations promote the proliferation of cancer cells, not expressing the CaSR, then the sequestration of this quantity should be able to block the replicative pressure. The hypothesis of a possible use of CPPs as anticarcinogens in the intestine, therefore, could be an innovative approach to block the carcinogenic hyperproliferation of cells, contributing to stop of tumor progression.

Therefore the aim of the present PhD thesis was:

1- to explore the possible modulation of cell proliferation and apoptosis by CPPs and by the CPP-mediated calcium influx in differentiated HT-29 cells, identifying the calcium channels involved, mainly such as the L-type calcium channels known to be expressed in this cell line (Larsson et al. 1998; Zawadzki, et al. 2008). The same studies were carried on in undifferentiated and differentiated HT-29 cells, in order to verify the possibility that CPPs, due to their property to complex calcium ions, may behave
also as EGTA, the known calcium chelating agents, affecting the extracellular medium and influencing cell functions (Barbiero, et al. 1995).

2- to clarify whether the biological effect of CPPs in Caco2 cell line was also due to the modulation of L-type calcium channels, or to an interaction with the TRPV6 channel, the main responsible of the transcellular transport of calcium in the intestinal epithelium (Peng et al. 1999).
2.1 REFERENCES


3. Theme 1
In recent years great interest has been directed to bioactive compounds, molecules naturally present in foods which can exert physiologically relevant effects at multiple levels in the body, such as at cardiovascular, nervous, gastrointestinal and immune level (Meisel 2004). The goal of all the studies devoted to discover new bioactive molecules in food is to improve human health simply by consuming a “normal diet” which naturally contains a consistent amount of these bioactive molecules, or by using them as additives in food.

Milk and dairy products are known sources of bioactive peptides; the major protein group of dairy products is casein. Casein does not display any bioactivity, being a reservoir of amino acids for our health, but peptides coming from the in vitro and/or in vivo casein digestion can display particular bioactivities. Among these peptides, casein phosphopeptides (CPPs) represent a family characterized by the ability to bind and solubilise cations, especially calcium (Berrocal et al. 1989).

The CPP ability in binding calcium and activate the mineral entry into the intestinal cells has been observed in several in vitro studies (Ferraretto et al. 2003; Ferraretto et al. 2001; Gravaghi et al. 2007). Moreover, in the last years new potentialities for CPPs such as the modulation of cell viability, i.e. proliferation and apoptosis, have been demonstrated in different human cell cultures (Hartmann et al. 2000; Meisel and Gunther 1998).

This is of great importance when considering that abnormal cell proliferation and apoptosis inhibition is often associated to pathological conditions as inflammation and tumor (Brown and Attardi 2005). It was also showed that the mechanisms of entry of calcium ions into cells have been associated with cell growth in normal and tumor tissues (Munaron et al. 2004).

More recently, it was demonstrated that in HT-29 cells, as well as in a primary human colon cancer cell line (AZ-97), the activation of voltage-activated L-type calcium channels, which mediate the calcium influx according to the depolarization state of the cell, is correlated to apoptosis and their blockade may promote the growth of colon cancer cells (Zawadzki et al. 2008).

There is, in fact, clear evidence that L-type channels are present in enterocytes; this channel is present in the apical membrane of rat intestine, its expression results more elevated in distal jejunum and proximal ileum, where the calcium absorption is conventionally considered to be higher, about 85%, and to occur by the paracellular route, while at duodenum, where the active transport occurs, the TRPV6 channel is predominant. The two channels, apart for their localization, differ in their operating mode: i) Cav1.3 is activated and mediate the calcium transport under depolarizing conditions, TRPV6 channel is activated by hyperpolarization; ii) Cav1.3 is sensitive to the same agonist/antagonists for L-type calcium channels here used, while TRPV6 is not (Morgan et al. 2003). Also a recent review supports the idea that this two channels have a complementary roles in the regulation of intestinal calcium absorption as depolarization and repolarization of the apical membrane occur during and between digestive periods, respectively (Kellett 2011).
Based on this evidence and the role attributed to calcium in the development and/or regression of colon cancer (Whitfield et al. 1995), the aim of this study is to explore the possibility that the CPP-mediated calcium influx in differentiated HT-29 cells could involve the L-type calcium channels known to be expressed in this cell line (Zawadzki et al. 2008) and to study the possible correlated modulation of cell proliferation and apoptosis.

All the experiments were done at physiological condition, the calcium concentration present in the growing medium, and at calcium overload. The overload of calcium used (6 mM) were chosen in order to mimic the situation at intestinal lumen after a meal, since it is reported in literature that in this particular condition the extracellular calcium concentration can reach values as high as 10 mM (Bronner 2003). Since tumor cells grow in 2 mM calcium concentration, while differentiated cells require small amounts of calcium ions to survive (Whitfield et al. 1995), (0.4 mM calcium in RPMI 1640 medium), the experiments with differentiated cells were carried on both at 2 mM and 6 mM calcium, in order to compare results with undifferentiated cells and to reproduce more than one step in the increasing calcium concentration after a meal.
3.1 Materials and Methods 1
MATERIALS AND REAGENTS

Cell culture media, L-Glutamine, antibiotic-antimycotic solution, trypsin-EDTA solution, Bay-K8644, Nifedipine, Nimodipine and all other reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS) was from EuroClone Ltd (West Yorkshire, UK). Fura-2 acetoxymethyl ester (Fura-2/AM) was from Calbiochem (La Jolla, CA, USA). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, DiBAC₄(3) was obtained from Molecular Probes (Eugene, OR, USA).

CELL CULTURE

HT-29 cell line

**Maintenance in culture.** The human colon adenocarcinoma-derived HT-29 cell line (BS TCL 132) was supplied by Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy). These cells, after thawing, are routinely grown in DMEM (Dulbecco’s Modified Eagle Medium) culture medium with high D-glucose content (4.5g/L). The cells are cultured in 75cm² plastic flasks (Costar, Concorezzo, Italy) and kept at 37°C in a 5% CO₂-95% air atmosphere. The DMEM medium is supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-Glutamine, 0.1 mg/L streptomycin, 1·10⁵U/L penicillin, 0.25 mg/L amphotericin B. Medium was periodically changed. When confluent, the cells are detached from the growth substrate through enzymatic digestion (Trypsin-EDTA 0.05-0.02%) of the adhesion-mediating proteins and then subcultivated.

**Cultivation in RPMI-1640.** After detaching by trypsin-EDTA (0.05-0.02%) cells were resuspended in RPMI-1640 (Roswell Packard Memorial Institute) growth medium and seeded in a new 75 cm² flask, where they were maintained till the following passage. The RPMI-1640 medium has a low D-glucose content (2g/L) and it is supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-Glutamine, 0.1 mg/L streptomycin, 1·10⁵U/L penicillin, 0.25 mg/L amphotericin B. The medium was changed twice a week.

When a confluence status is reached, the cells are detached from the substratum and subcultivated for about 20 passages.

Apart the D-glucose content, the DMEM and RPMI-1640 media differ for inorganic salt composition and concentration, aminoacids and vitamins.
METHOD OF DIFFERENTIATION OF THE CELL LINE HT-29

HT-29 CELLS GROWTH IN DMEM MEDIUM

100% CONFLUENCY

TRYPsin-EDTA (0.05%-0.02%)

REMOVAL OF DMEM MEDIUM

SEEDING IN RPMI 1640 MEDIUM

SUB-CULTURE OF HT-29 RPMI CELLS

Figure 1. Schematic diagram of the growth and differentiation of the HT-29 cell line (Cosentino et al. 2010).
There have been utilized a commercial CPP mixture kindly provided by DMV (DMV International, Netherlands).

This CPP preparation was constituted by a tryptic derivative of the bovine casein and is composed by peptides coming from the proteolitic cleavage of \( \alpha_s1 \) and \( \beta \)-casein. The use of a CPP mixture, as the CPP DMV, was justified by the fact that, as a result of the casein enzymatic gastrointestinal digestion, a mixture of different peptides is normally formed.

The commercial preparation present a molecular weight of 2500 Da. All the chemical characteristics are reported in Table 1.

All the experiments were conducted using the solubilised form of the preparation (200 mg of CPP in 500 µL of double distilled water).

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<th>CPP DMV (CE 90 CPP III)</th>
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<tr>
<td>CPP content</td>
<td>90.5%</td>
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<tr>
<td>( \text{Ca}^{2+} ) content</td>
<td>0</td>
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<tr>
<td>P content</td>
<td>3.7%</td>
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<tr>
<td>NP ( \cdot 1 ) (molar ratio)</td>
<td>3.7</td>
</tr>
<tr>
<td>Ser P( \cdot 1 ) (molar ratio)</td>
<td>0.85</td>
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*Table 1.* Chemical composition of the CPP preparation. The reported data was supplied by the producer.
CYTOPLASMIC CALCIUM CONTENT MISURATION AT SINGLE CELL LEVEL

Video microscopy experiments allowed to evaluate the CPP ability to induce transient increases of the \([\text{Ca}^{2+}]_i\) in HT-29 cells. Cells were seeded onto sterilized 0.13-0.17 thick cover glasses (Ø = 24mm) in 35 mm Petri dishes, at the density of \(2.6 \times 10^4\) cells/cm². Two days after seeding, sub-confluent cells were processed. Following the medium removal, cells were loaded with 2µM FURA-2/AM and 2µM Pluronic F-127 (an anionic surfactant that favours the Fura-2/AM dispersion), for 15 minutes at 37°C in a standard Krebs-Ringer-HEPES buffer (KRH SD) containing in mM: 125 NaCl, 5 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 HEPES, 2 CaCl₂, pH 7.4, according to the procedure already described (Cosentino et al. 2010; Ferraretto et al. 2001) For the analysis it was used a inverted epifluorescent microscope (Nikon, TE 200) equipped with a thermostatic perfusion chamber (PDMI-2 TC-202 A, Medical System Corporation Harvard Apparatus, Holliston, MA, USA). The cover glass was mounted within the thermostatic chamber with 2 mL KRH SD and kept at 37°C. The analysis was carried out within a delimited region of the glass (cellular field) containing an average of 80 cells, selected by the software. The sample was excited with light coming from a Xenon lamp, which wavelengths is selected through the connected monochromator. An automatic device alternate between the two excitation wavelengths and the excitation light reaches the sample through a 40x oil immersion objective with NA= 1.3 (Nikon Corporation, Tokyo, Japan). The emitted fluorescence (\(\lambda = 510\)nm) was recorded at 1-2 seconds intervals by a intensified CCD camera (Extended Isis, Photonic Science, UK). The system recorded the emission at 510 nm following excitation at both 340 and 380 nm, it calculated the ratio between the two fluorescence intensities and the images of each cell of the field were acquired and analysed, previous background value subtraction, using a data acquisition and analysis system (High Speed Dynamic Video Imaging Systems – Quanticell 700, Applied Imaging). The free calcium ions concentration were calculated from the ratio of the emitted fluorescence intensity following excitation at 340 and 380 nm, referring to a calibration curve obtained using standard solutions of free Fura-2, in presence of EGTA 5 mM (that totally chelates calcium), and of complexed Fura-2 in presence of saturating concentration of \(\text{Ca}^{2+}\) (5mM), applying the Grynkiewicz equation (Grynkiewicz, et al. 1985).
EVALUATION OF THE EFFECTS OF INHIBITORS/ACTIVATORS OF THE VOLTAGE OPERATED L-TYPE CALCIUM CHANNELS

To evaluate if the mechanisms of action of the CPPs was correlated to the activity of the L-type voltage operated calcium channels, and thus that the CPP-induced intracellular calcium increases were mediated by their activation, cells were pre-treated with an agonist and two antagonists of these channels, before the CPP administration, to evaluate the consequent effects.

The (±)Bay-K8644, is a L-type Ca\(^{2+}\)-channel activator widely used to evaluate the entity of the calcium influxes through the L-type channels in various cell lines, and among them the HT-29 (Larsson et al. 1998).

Nimodipine and Nifedipine, two dihydropyridine derivatives, are L-type Ca\(^{2+}\)-channel antagonists that have been shown to be able to promote an antiproliferative effect in epidermal carcinoma-derived epithelial cells (Larsson et al. 1998). Both antagonists were solubilised in DMSO and administered to cells at the concentration of 30 µM to HT-29 cells. In fact, HT-29 cells have been demonstrated to express the L-type Ca\(^{2+}\)-channels in the apical membrane.

The treatments with these compound were carried out to evaluate their effects on the CPP-induced intracellular calcium increases, in video microscopy experiments.

Bay-K8644, previously solubilised in DMSO, was administered to HT-29 RPMI cells at 400 nM concentration. Both Nimodipine and Nifedipine were solubilised in DMSO and administered to differentiated HT-29 cells at 30 µM concentration. In Video-imaging experiments, it was primarily evaluated the cell responsiveness to CPPs, then after a pre-incubation with the agonist or the antagonists, cells were stimulated with a second CPP dose. LaCl\(_3\), known to inhibit Ca\(^{2+}\) entry and cause an irreversible decrease in [Ca\(^{2+}\)]\(_i\) (Tsunoda, et al. 1990), was administered to cells at 250 µM concentration after treatment with CPPs.

Cells were submitted to the following stimulation schedules:

1) 1280µM CPP
2) washes with KRH SD with 2 mM CaCl\(_2\)
3) 30 µM Nimodipine or 30 µM Nifedipine or 400nM (±)-Bay-K8644
4) 1280 µM CPP
5) 100 µM ATP in the case of absence of response, with the aim to verify the viability of the cell populations
CELL PROLIFERATION EVALUATION

Cell Proliferation ELISA, BrdU chemiluminescence

The effects of different treatments on cell proliferation were evaluated through the measurement of the bromodeoxyuridine (BrdU) cell incorporation, a thymidine analogous, which incorporates in the nuclear DNA, during the S phase of the cell cycle, of actively dividing cells. Confluent cells were detached from the adhesion substrate by trypsin-EDTA (0.05% - 0.02%) treatment, and seeded in a black 96 wells plate at the density of 1-10^4 cells/well, in a final volume of 200 µL. After 24 hours the medium was changed and the cells were treated for 24 h with either 1280 µM CPP or 500 µM EGTA in foetal bovine serum free media, in the presence of 0.4 - 2 - 6 mM CaCl₂. The effect of 400 nM Bay-K8644, 30 µM Nimodipine, 30 µM Nifedipine in the presence of 0.4 mM and 2 mM CaCl₂ was determined following the same procedure.

At the end of the incubation, cells were submitted to a 2 h pulse with bromodeoxyuridine (BrdU) and BrdU incorporation into DNA was quantified by the chemiluminiscent immunoassay (Roche Applied Science, Milan, Italy) following the manufacturer’s instructions. The photon emission was measured by a photomultiplier (VICTOR² Wallac 1420 multilabel counter, PerkinElemer).

APOPTOSIS EVALUATION

Apo-ONE® assay

The Apo-ONE® assay (Promega, Madison, WI, USA) is a practical tool to measure the activities of caspases (Cysteine protease Aspartate) 3 and 7, which are known to act as key effectors in the apoptosis of mammalian cells (Vaux and Strasser 1996). The Apo-ONE® is based on the ability of Caspase -3 and -7 to hydrolyse the artificial substrate Z-DEVD-R110 [Rhodamine110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)]. The Z-DEVD-R110 is a profluorescent molecule. If pro-apoptotic stimuli cause the activation of Caspase -3 and -7, these operate the cleavage of the site of the C-terminal aspartate residue sequence DEVD (Asp-Glu-Val-Asp) and the removal of the substrate peptide DEVD, with gradual release of fluorophore rhodamine 110, which emits fluorescence intensity (λex= 499 nm, λem= 521 nm). The intensity of fluorescence produced is proportional to the activity of Caspase -3 and -7 of the sample and is quantified and compared with the fluorescence emitted by a concentration of standard note rhodamine110.

Cells (1x10⁴ cells/well), plated in a Microtiter plate (96-well, Greinerbio-one, Cellstar, Frickenhausen, Germany), were treated for 24 h with either 1280 µM CPP or 500 µM EGTA in foetal bovine serum free media, in the presence of 0.4 - 2 - 6 mM CaCl₂.
At the end of the incubation are added to each well 100 μl of reagent, established by the Caspase Substrate and Apo-ONE® Caspase-3/7 Buffer, 1:100 ratio. After a short wave (300-500 rpm, 37° C, 30 seconds) the cells are left at room temperature, strictly in the dark. The fluorescence emission at 530 nm, with excitation at 485 nm, was monitored between 1 h and 7 h by VICTOR² Wallac 1420 Multilabel Counter (Perkin Elmer, Beaconsfield, UK).

Dapi staining
The DAPI (4-6 diamino-2-phenylindole-) is a dye that selectively forms fluorescent complexes with DNA and showing high specificity for regions rich in AT, IC and AU. Based on these properties DAPI is an extremely useful tool in the cytochemical investigations: when it binds to DNA, absorbs at a wavelength of 340 nm and emits at a wavelength of 488 nm, with fluorescence intensity about 20 times greater than the dye not complexed to nucleic acids. At a time when the cell membrane of apoptotic cells is impaired, greater amount of DAPI penetrates into the cell and promotes the increase of staining directly proportional to the concentration of DNA present. The different morphology of apoptotic nuclei, due to a different chromatin condensation or fragmentation, may affect the identification of stained cells with DAPI.

Cells (5x10⁴ cells) seeded on glass coverslips were treated for 24 h with either 1280 μM CPP or 500 μM EGTA in foetal bovine serum free media, in the presence of 0.4 - 2 - 6 mM CaCl₂. The same procedure was adopted to study the effect of 400 nM Bay-K8644; 30 μM Nimodipine; 30 μM Nifedipine in the presence of 0.4 mM and 2 mM CaCl₂ in HT-29 cells. After the incubation, cells were fixed in 70% ethanol for 15 min at −20°C. Then they were stained with 0.4 mg/µL 4’,6-diamidino-2-phenylindole (DAPI) for 5 min and the morphology of the nuclei was examined by Olympus microscope BX50 (Olympus, Tokyo, Japan) at excitation and emission wavelengths of 358 and 460 nm, respectively. The results are expressed as percentage of apoptotic nuclei with respect to the total nuclei present in each examined cellular fields, for a total of 12 cellular fields analyzed for each treatment in a single experiment.
MEASUREMENT OF MEMBRANE POTENTIAL

DiBAC₄(3) belongs to a class of anionic slow potential-sensitive dyes (Plasek and Sigler 1996) and has been shown to respond to membrane depolarization with an increase in fluorescence resulting from the increased intracellular concentration and accumulation in intracellular lipid-rich compartments (Postma, et al. 1996). Differentiated HT-29 cells (2.5x10⁴ cells/well), plated in a Microtiter plate (96-well, Greinerbio-one, Cellstar, Frickenhausen, Germany), were loaded with 500 nM DiBAC₄(3) in KRH solution. After 15 min of incubation at 37°C and 300 rpm in a Thermomixer (Eppendorf, Hamburg, Germany), fluorescence was recorded (λex 490 nm, λem 510 nm), and here reported as “before stimulus”. Then, either 1280 μM CPP DMV, or 1 μM Gramicidin, or an hyperpolarizing KRH solution (5 mM KCl, 0 mM NaCl) was added and fluorescence immediately recorded and here reported as “after stimulus”.

STATISTICAL ANALYSIS

Statistically significant differences between two mean values were established by the Student’s $t$ test, independent two population $t$ test, performed with Origin 6.0 (a $P$ value < 0.05 was considered significant).
3.2 Results 1
**CPP EFFECTS ON PROLIFERATION RATE IN UNDIFFERENTIATED/DIFFERENTIATED HT-29 CELLS**

In undifferentiated HT-29 cells the proliferation rate both at physiological \([\text{Ca}^{2+}]_o\) (2 mM) in the growth medium and in calcium overload (6 mM) was not affected by the cell incubation with CPPs (a dose at which they are demonstrated to exert the maximal bioactivity (Cosentino et al. 2010; Gravaghi et al. 2007) or EGTA (Figure 2A).

In differentiated HT-29 cells the proliferation rate, measured at 0.4 mM \([\text{Ca}^{2+}]_o\), which represents the physiological calcium concentration in the growth medium, and at 2 and 6 mM \([\text{Ca}^{2+}]_o\), calcium overload conditions, was increased by CPPs above all at 2 mM \([\text{Ca}^{2+}]_o\) (p< 0.05). EGTA determined a strong reduction of the proliferation rate at 0.4 mM \([\text{Ca}^{2+}]_o\), an event not observed at 2 mM \([\text{Ca}^{2+}]_o\), while at 6 mM \([\text{Ca}^{2+}]_o\) the proliferation rate was reduced by 43% (p< 0.05), (Figure 2B).

**Figure 2.** Assessment of the proliferation rate using bromodeoxyuridine assay in HT-29 cells undifferentiated (A) and differentiated (B), after administration of 1280 µM CPP and 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments. Symbols indicate a statistically different value from control (*p < 0.05, #p< 0.01).
CPP EFFECTS ON APOPTOSIS IN UNDIFFERENTIATED/DIFFERENTIATED HT-29 CELLS

In undifferentiated HT-29 cells the apoptotic activity was increased by CPP administration both at 2 mM [Ca\(^{2+}\)]\(_o\) (caspase 3/7 activity and apoptotic nuclei staining, Figure 3A and 3B), and at 6 mM [Ca\(^{2+}\)]\(_o\) (apoptotic nuclei staining, Figure 3B). The administration of EGTA similarly induced an increase in the apoptotic activity both at 2 and 6mM [Ca\(^{2+}\)]\(_o\) (apoptotic nuclei staining, Figure 6B). The apparent different results obtained by mean of the two different apoptotic activity measurements could be due to the chronologically different events in the apoptosis, since the activation of caspases precedes the nuclear fragmentation and condensation (Vaux and Strasser 1996).

Figure 3. Assessment of apoptosis in undifferentiated HT-29 cells by Apo-One assay (A) and by DAPI staining (B) following administration of 1280 µM CPP and 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments. Symbols indicate a statistically different value from control (*p < 0.05, #p< 0.01).
The apoptotic activity of differentiated HT-29 cells was increased by EGTA at all the \([\text{Ca}^{2+}]_o\) considered, except at 6 mM \([\text{Ca}^{2+}]_o\), when the caspase 3/7 activity decreases, while CPP administration was without any effect (Figure 4A and 4B).

**Figure 4.** Assessment of apoptosis in differentiated HT-29 cells by Apo-One assay (A) and by DAPI staining (B) following administration of 1280 µM CPP and 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments. Symbols indicate a statistically different value from control (*p < 0.05, #p< 0.01).

Images of Dapi stained nuclei (Figure 5 and 6) before and after CPP and EGTA administrations are meaningful to understand these results.

Undifferentiated tumor HT-29 cells are not sensitive to calcium overload (6 mM), in that no differences were observed either in the proliferation rate (see Figure 2A) and in apoptosis activity compared to control cells (Figure 5A and 5D), probably because they lack part of the molecular machinery necessary to sense the variation in the extracellular calcium concentration able to modulate differentiation and apoptosis (Whitfield 2009). Differences were observed with the treatment with CPPs or EGTA both in standard condition 2 mM, and in calcium overload (6 mM): while the proliferation rate does not change (see Figure 2A), apoptosis activity increased (Figure 5B, 5C, 5E and 5F), probably due to the CPP and EGTA action of binding calcium ions, thus subtracting the proliferative stimulus for tumor cells, without the possibility for them to differentiate (Whitfield 2009).
Figure 5. Images of DAPI stained nuclei in undifferentiated HT-29 cells. Photographs of: control cells (Panel A), 1280 μM CPP treated cells in presence of 2 mM [Ca$^{2+}$]$_o$ (Panel B), 500 μM EGTA treated cells in presence of 2 mM [Ca$^{2+}$]$_o$ (Panel C), control cells in presence of 6 mM [Ca$^{2+}$]$_o$ (Panel D), 1280 μM CPP treated cells in presence of 6 mM [Ca$^{2+}$]$_o$ (Panel E), 500 μM EGTA treated cells in presence of 6 mM [Ca$^{2+}$]$_o$ (Panel F). Magnification was 40x.

In differentiated HT-29 cells, after the addition of 500 μM EGTA in 0.4 mM [Ca$^{2+}$]$_o$, cell nuclei appeared smaller and rounded (Figure 6B), a classical morphological sign of apoptosis, compared to control cells (Figure 6A), accordingly to the drastic fall observed in their proliferative rate (see Figure 2B). Probably under this experimental condition EGTA has subtracted all the calcium necessary to cells for surviving. When 500 μM EGTA was administered in 2 mM [Ca$^{2+}$]$_o$ the appearance of Dapi stained nuclei (Figure 6C) was not different vs control cells, in accordance with a normal proliferative rate and a slight increase in the apoptotic activity (see Figure 3). CPPs, though displaying a strong ability to bind calcium ions, did not behave as a cytotoxic agent neither in 0.4 mM [Ca$^{2+}$]$_o$ (Figure 6D) nor in 2 mM [Ca$^{2+}$]$_o$ (data not shown). At 6 mM [Ca$^{2+}$]$_o$, a condition in which a lot of cells were not viable (Figure 6E), the CPP addition reversed the cells to the control condition (Figure 6F), confirming previous observations about its role in maintaining calcium ions in a soluble form (Berrocal et al. 1989), on the contrary of EGTA.
Figure 6. Images of DAPI stained nuclei in differentiated HT-29 cells. Photographs of: control cells (Panel A), 500 μM EGTA treated cells in presence of 0.4 mM [Ca\(^{2+}\)]\(_{o}\) (Panel B), 500 μM EGTA treated cells in presence of 2 mM [Ca\(^{2+}\)]\(_{o}\) (Panel C), 1280 μM CPP treated cells in presence of 0.4 mM [Ca\(^{2+}\)]\(_{o}\) (Panel D), control cells in presence of 6 mM [Ca\(^{2+}\)]\(_{o}\) (Panel E), 1280 μM CPP treated cells in presence of 6 mM [Ca\(^{2+}\)]\(_{o}\) (Panel F). Magnification was 40x.
EFFECT OF AGONIST/ANTAGONIST OF VOLTAGE OPERATED L-TYPE CALCIUM CHANNELS ON PROLIFERATION RATE AND APOPTOSIS IN DIFFERENTIATED HT-29 CELLS

CPPs are able to induce $[\text{Ca}^{2+}]_i$ rise only in differentiated intestinal cells (Cosentino et al. 2010), thus the hypothesis that the CPP-induced increment in the proliferation rate of differentiated HT-29 cells could be associated to the influx of calcium ions activated by the same CPPs. It’s known that an influx of calcium through voltage operated L-type calcium channels have a mitogenic effect (Munaron et al. 2004), therefore experiments were performed in differentiated HT-29 cells with the use of agonist and antagonists of this channels. The possibility that the activity of these channels could affect the cell proliferation rate and apoptosis, as already described in different in vitro cell models (Larsson et al. 1998; Yoo, et al. 1997; Yoshida, et al. 2003; Zawadzki et al. 2008) was studied at 0.4 mM and at 2 mM $[\text{Ca}^{2+}]_o$. The cell treatment with the agonist Bay-K8644 increased the proliferation rate (Figure 7A). Nimodipine and Nifedipine, which act as L-type calcium channel antagonists (Fox and Green 1986; Larsson et al. 1998), induced a significant reduction in the proliferation rate together with an increase in the apoptosis level (Figure 7B).

Figure 7. Effect of agonist and antagonists of L-type $\text{Ca}^{2+}$ channels in differentiated HT-29 cells. Proliferation rate (Panel A) and apoptotic activity determined by DAPI staining (Panel B) of: control cells (Crt, 100%), cells treated with either 400 nM Bay-K8644, or 30 µM Nifedipine, or 30 µM Nimodipine in presence of 0.4 mM and 2 mM $[\text{Ca}^{2+}]_o$. Each bar represents the mean ± S.D. of 3 analogous experiments. Symbols indicate a statistically different value from Crt (*$p < 0.05$, #$p < 0.01$).
INTERACTION OF CPPs WITH L-TYPE CALCIUM CHANNELS

Differentiated HT-29 cells were pre-treated with agonist or antagonists of L-type calcium channels and [Ca^{2+}]_i changes were recorded by computerized video microscopy after CPP administration.

The behaviour of these cells when stimulated with Bay-K8644 in presence of 2 mM [Ca^{2+}]_o is reported in Figure 8A. The intracellular calcium rises recorded indicates the presence of active L-type Ca^{2+} channels in the cell population here used, according to the literature (Ferrante, et al. 1989; Larsson et al. 1998).

The administration of the same agonist, Bay-K8644, in CPP responsive cells produced a higher response to the subsequent CPP administration (Figure 8B), which accounts for a 100% increase in the [Ca^{2+}]_i and a 50% increase in the percentage of responsive cells (Table 2).

![Figure 8](image)

**Figure 8.** Effect of Bay-K8644 on CPP induced calcium rise in differentiated HT-29 cells. (A) 400 nM Bay-K8644 was administered in 2 mM [Ca^{2+}]_o. (B) 1280 μM CPP administration, cell washing and restoration of initial conditions (time axis break), followed by 400 nM Bay-K8644 and subsequently 1280 μM CPP. Each line in the graph refer to a single cell behavior. Each graph is representative of at least 5 analogous experiments.
Both antagonists, Nifedipine and Nimodipine, were able to totally or partially reduce the CPP-induced calcium rise in HT-29 RPMI cells, as shown in Figure 9A and 9B. The percentage of cells which did not responded to CPP administration after Nimodipine or Nifedipine treatment was almost the same and accounts for about 36-38%. The remaining 64-62% of the cells responded to CPPs but with a marked reduction in the percentage of responsive cells (minus 45%) and in the intracellular calcium concentration (minus 52%) in the case of Nimodipine (Table 2), while in the case of Nifedipine a marked reduction (minus 47%) was observed only in the intracellular calcium concentration (Table 2).

Figure 9. Effect of Nifedipine and Nimodipine on CPP induced calcium rise in differentiated HT-29 cell.
(A) 1280 μM CPP administration, cell washing, 30 μM Nifedipine incubation (10 min, time axis break), followed by 1280 μM CPP and 1 μM ATP, as a marker of full cellular viability.
(B) 1280 μM CPP administration, cell washing, 30 μM Nimodipine incubation (10 min, time axis break), followed by 1280 μM CPP and 1 μM ATP.
Each line in the graph refers to a single cell behavior. Each graph is representative of at least 5 analogous experiments.
Table 2. Statistical analysis of the effects on [Ca^{2+}]_i produced by CPP administration after cell treatment with Bay-K8644, Nimodipine and Nifedipine. The [Ca^{2+}]_i rise was calculated for each single cell by subtracting the baseline from the peak value after CPP administration and averaging for all the analyzed cells. The percentage of responsive cells was the percentage of cells which responded to CPP administration with [Ca^{2+}]_i increments equal to or above 20 nM. Each value represents the mean ± SD of 4 analogous experiments. Symbols indicate a statistically different value between pre and post-agonist/antagonist administration (*p<0.05, #p<0.01).

<table>
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<tr>
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<th>Pre-agonist/antagonist</th>
<th>Post-agonist/antagonist</th>
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<tr>
<td></td>
<td>% Responsive cells</td>
<td>Δ [Ca^{2+}]_i nM</td>
</tr>
<tr>
<td>Bay-K8644</td>
<td>67±20.8</td>
<td>65.5±17</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>91±9</td>
<td>92±48</td>
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<tr>
<td>Nifedipine</td>
<td>61±23</td>
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A further demonstration of the involvement of the L-type Ca^{2+} channels came from the use of La_{2}Cl_{3}, an inorganic inhibitor of calcium entry (Tsunoda et al. 1990), which when pre-administered to cells abolished the CPP bioactivity (Figure 10).

![Figure 10](image_url)

**Figure 10.** Changes in the intracellular calcium concentration when cells were stimulated with 1280 μM CPP, washed and incubated with 250 μM La_{2}Cl_{3} (5 min) and finally treated with 1280 μM CPP. Each line in the graph refer to a single cell behavior. Each graph is representative of at least 5 analogous experiments.
The membrane potential of differentiated HT-29 cells was measured by the use of DiBAC$_4$(3), a probe whose fluorescence increases in depolarized conditions and decreases during hyperpolarization.

CPPs increased the fluorescence activity (plus 16%) at the same entity of Gramicidin (plus 13%), a known depolarizing agent (Bronner and Landry 1991), while the hyperpolarizing KRH solution decreased fluorescence (minus 34%) (Figure 11).

**Figure 11.** Effect of CPP administration on membrane potential in differentiated HT-29 cells. Black bars represent fluorescence intensity of cells loaded with 500 nM DiBAC$_4$(3) before the additions of stimuli (100%). White bars represent the change in fluorescence intensity (percentage vs “before stimuli”) of control cells (Crt) and cells treated with: CPP (1280 μM), Gramicidine (1 μM) and hyperpolarizing KRH (5 mM KCl). Each bar represents the mean ± S.D. of 4 analogous experiments. Statistically different value vs “before-stimuli” condition (*p<0.01).
3.3 Conclusions 1
In the intestinal crypts, the concentration of extracellular calcium can modulate proliferation and apoptosis, cellular events related to the maintenance of a differentiated phenotype and/or development of a tumor (Whitfield et al. 1995). In fact, the intestinal cells survive with concentrations of 0.05-0.1 mM calcium, and when an excess of calcium reaches the intestinal lumen, for example after a meal, the cell proliferation stops and the expression of a differentiated phenotype, culminating in apoptosis, takes place. The molecular mechanisms underlying this transformation sees the involvement of two main mechanisms: the Wnt mechanism and the CaSR (Whitfield 2009).

Enterocytes that undergo neoplastic transformation lose their ability to respond to the differentiating stimulus represented by a high concentration of extracellular calcium, due to decreased or absent expression of CaSR. Then they become resistant to apoptosis and are actively proliferating (Chakrabarty et al. 2003; Chakrabarty et al. 2005). In colon adenocarcinoma cells, increased extracellular calcium concentration with the diet results in the stimulation of proliferation, while the same concentration of extracellular calcium has a pro-apoptotic effect on surrounding healthy cells.

Milk and dairy foods are the most bioavailable source of calcium, due to the presence of bioactive peptides such as CPPs which keep it in a soluble form. Hence the hypothesis that the CPPs, already shown to be able to increase the intracellular concentration of calcium in \textit{in vitro} models of intestinal cells HT-29 and Caco2 (Cosentino et al. 2010), may also affect cellular processes of proliferation and apoptosis in undifferentiated and differentiated HT-29 cells.

In undifferentiated tumor HT-29 cells CPPs do not alter the proliferation, but increase apoptosis, and this result is also obtained by EGTA, a known calcium chelator. Probably they act by the same mechanism: they bind the extracellular calcium, lowering its concentration, thus exerting a stimulus in the sense of reducing the proliferative rate, but since these cells lack the necessary mechanisms to differentiate go into apoptosis (Figure 12). However, the usage of EGTA as anti-proliferative agent in tumor regions is inadequate due to its toxicity for the neighbouring differentiated cells, while CPPs never display a cytotoxic activity at the concentration here used (see DAPI images).
Figure 12. In cancer cells the presence of extracellular calcium increased proliferation, promoting tumor progression. The chelating action of CPPs against the extracellular calcium induces apoptosis, suggesting that these peptides may be considered as probable anticarcinogens.

In contrast in differentiated HT-29 cells CPPs do not affect apoptosis, but increase the proliferation with a maximum effect at 2 mM [Ca²⁺]₀. This is in agreement with previous studies, which showed that CPPs induce an uptake of calcium ions in differentiated HT-29 cells in presence of extracellular calcium ions and with a precise dose response relationship (Gravaghi et al. 2007), according to which their ability, due to the formation of aggregates with calcium ions, is higher at 2 mM compared to 6 mM [Ca²⁺]₀. EGTA reduces the proliferation at physiological [Ca²⁺]₀, this is because it subtract the calcium needed by cells to survive, in fact this event does not happen at 2mM [Ca²⁺]₀, while at 6 mM [Ca²⁺]₀, the effect is added to the toxicity of such a high concentration of calcium. EGTA also increases apoptosis of these cells, especially at physiological and at 6 mM [Ca2+]₀, and this is in analogy with the reduction of the proliferative rate (see the images of DAPI staining). These results indicate a large difference between CPPs and EGTA on differentiated intestinal cells because, although it is able to bind calcium ions, CPP is not toxic at physiological [Ca²⁺]₀ or in the presence of an excess of [Ca²⁺]₀. Therefore CPPs protect differentiated cells from the toxicity caused by calcium overload and promote their proliferation, while induces apoptosis in cancer cells.

These results revealed a potential role for CCPs as nutraceuticals and/or functional foods, a role distinct from that of mineral carriers, but still important.

In fact, Wright and colleagues say that if the molecules with inhibitory activity to apoptosis are considered tumor promoters (Wright et al. 1994), the peptides that show a capacity to induce apoptosis may be considered as possible anticarcinogens (Meisel...
and FitzGerald 2003). Previous studies have demonstrated an anti-proliferative effect of the yogurt fraction on cultured mammalian cells Caco2 and IEC-6 (Ganjam et al. 1997), an inhibition of peripheral blood cell lymphocyte proliferation (Meisel and Gunther 1998), an influence on intestinal cell kinetics by casein peptides generated by dairy starter cultures (MacDonald, et al. 1994). Taken together, the experimental evidences here presented, whenever confirmed in further in vitro models and in vivo, could account for an anticancer role of CPPs.

To understand the mechanisms by which CPPs promote intracellular calcium entry and, at the same time, the proliferation in differentiated HT-29 cells experiments were conducted in the presence of agonists and antagonists of L-type calcium channels, known to exert pro/anti-proliferative effects in various cell cultures in vitro (Larsson et al. 1998; Yoo et al. 1997; Yoshida et al. 2003; Zawadzki et al. 2008). Results here presented demonstrate an interaction between CPPs and these calcium entry channels in differentiated HT-29 cells.

Besides, we cannot exclude the contribution, and the interaction of CPPs with other channels, since the correlation between ion channels and cancer prevention/development is a matter of intensive studies as reported (Fraser and Pardo 2008; Lang, et al. 2005), and in recent years has shown that a given ion channel can influence both cell proliferation and apoptosis depending on the properties of the cells. Moreover, the activity of a channel may depend on the interplay with other channels.

The further result that CPPs, interacting with the L-type calcium channels, depolarize the membrane of intestinal cells is very impressive if we consider the recent study by Morgan and colleagues on the subunit Cav 1.3 of the L-type channels. Moreover, all the calcium present in our body comes from the diet and the principal digestion products, i.e. glucose, amino acids, and oligopeptides, are all depolarizing nutrients (Sharp and Debnam 1994; Ward and Boyd 1980).

After the ingestion of milk, CPPs is released by casein, the main protein fraction, and accumulate in ileum (Meisel and FitzGerald 2003), where they can associate calcium ions but can also interact with the other nutrients here present and deriving by the milk digestion as glucose from lactose and other peptides. The interaction of the different nutrients on calcium absorption constitutes an issue not completely understood, despite the recent findings here described (Morgan et al. 2007; Nakkrasae et al. 2010), which deserves further studies, but the results here presented underline the importance of consider CPPs as molecules for the intestinal health.
3.4 REFERENCES 1


Postma FR, Jalink K, Hengeveld T, Bot AG, Alblas J, de Jonge HR & Moolenaar WH 1996 Serum-induced membrane depolarization in quiescent fibroblasts: activation of...


4. Theme 2
All the calcium necessary to our body comes from the diet and it is absorbed mainly through two ways: the active transcellular route and the passive paracellular route. The highly calcium selective channel TRPV6, also designated as calcium transporter-1 or CaT1, is predominantly expressed in intestinal epithelial cells and has also been localized to human colon cancer (Caco2) cells (Fukushima, et al. 2009; Schoeber, et al. 2007) and regulating transcellular calcium absorption at intestinal level (Cui, et al. 2009; Taparia, et al. 2006; Wood, et al. 2001). The TRPV6 gene expression is regulated by 1,25-Dihydroxycholecalciferol (1,25(OH)2D3) and also by the extracellular calcium concentration, increasing at low and decreasing at high calcium ion concentrations, according to a saturation kinetic in the order of mM (Ramasamy 2006). However, the molecular mechanisms have not yet been identified but seems to exert a protective role when the calcium concentration in the intestinal lumen greatly increases reaching values above 6 mM, as observed after a calcium rich meal (Bronner 2003; Slepchenko and Bronner 2001), an event which could exert negative effects on the involved cells, explaining the rapid TRPV6 inactivation (Peng, et al. 2003).

Calcium deficiency in the diet is an important factor that increases the expression of TRPV6 in the duodenal mucosa of mice (Ko, et al. 2009). Short chain fatty acids likewise increase expression of TRPV6 and calcium absorption in the rat colorectal epithelium as well as in Caco2 cells (Fukushima et al., 2009). TRPV6 in the intestinal epithelium also appears to have a bearing on colon cancer development. Thus, TRPV6 expression is significantly enhanced in the Citrobacter rodentium-induced transmissible murine colonic hyperplasia model (Peleg, et al. 2010). Whereas in the normal colon TRPV6 is restricted to the apical membrane of absorptive enterocytes, in the hyperplasia model TRPV6 is also distributed to the proliferating zone of the colonic crypts, in which it occurs in the basolateral membrane and perinuclear area of many epithelial cells (Peleg, et al. 2010). When the animals are fed with a calcium-rich diet, the overexpression of TRPV6 in the hyperplasia model is reversed.

Increased levels of colonic TRPV6 are associated with early-stage colon cancer. Thus, TRPV6 has been found to be overexpressed in 66% of stage I tumors and in 17% of stage II tumors but is barely detectable in stages III and IV tumors (Peleg, et al. 2010). These results suggest that aberrant function of TRPV6 is associated with early colon carcinogenesis but not with frank malignancy (Peleg, et al. 2010). This inference is supported by experiments with the human colon carcinoma cell line Caco2, which constitutively express high levels of TRPV6.

Dairy food contain high amount of calcium in a soluble form and represent a source of molecules, distinct from lactose, able to affect the mineral absorption. In this context casein phosphopeptides (CPPs), coming from in vivo and/or in vitro bovine casein hydrolysis, due to their ability to bind and solubilise calcium ions, were a matter of numerous studies devoted to investigate a potential role for them in calcium absorption at the intestinal level (Cosentino, et al. 2010a; Cosentino, et al. 2010b; McDonagh and FitzGerald 1998).
Indeed, previous studies carried on at molecular level have demonstrated that differentiated Caco2 cells display a considerable calcium uptake following CPP administration, in contrast with the unresponsiveness of the undifferentiated counterparts (Cosentino et al. 2010b), besides the fraction of calcium bound to CPPs is directly involved in this event (Cosentino et al. 2010a).

Also the recent finding presented in the previous Theme 1 shown that in HT-29 cell lines CPPs modulate cell proliferation and apoptosis through the interaction of the L-type calcium channels.

Hence the aim of this study was to clarify whether the biological effect of CPPs in Caco2 cell line was also due to the modulation of L-type calcium channels, or to an interaction with the TRPV6 channel, and if this channel, like the L-type calcium channels, is involved in the modulation of proliferation and apoptosis.

The hypothesis of an interaction with a 1,25(OH)\(_2\)D\(_3\)-mediated channel like TRPV6 comes from the results obtained in previous experiments (Cosentino et al. 2010b) which showed that in undifferentiated Caco2 cells treated with 1,25(OH)\(_2\)D\(_3\) (and then driven to differentiation), the response to CPPs was greater than that of same cells differentiated according to the published protocol (Ferraretto, et al. 2007).

All the experiments of proliferation and apoptosis were done at physiological condition, the calcium concentration present in the growing medium, and at calcium overload. Moreover the TRPV6 channel was silencing with the RNA interference technique in order to verify a possible interaction with CPPs.
4.1 Materials and Methods 2
MATERIALS AND REAGENTS

Cell culture media, L-Glutamine, antibiotic-antimycotic solution, trypsin-EDTA solution and all other reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS) was from EuroClone Ltd (West Yorkshire, UK). Fura-2 acetoxymethyl ester (Fura-2/AM) was from Calbiochem (La Jolla, CA, USA).

CELL CULTURE

Caco2 cell line
The human colon adenocarcinoma derived Caco2 cell line (BS TCL 87) was supplied by Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy).
Cells are cultured in 75 cm² plastic flasks (Costar, Concorezzo, Italy) in EMEM (Minimum Essential Medium Eagle's) growth medium supplemented with 15% heat-inactivated foetal bovine serum (FBS), 1 mM sodium-pyruvate, 2 mM L-Glutamine, 0.1 mg/L streptomycin, 1·10⁵ U/L penicillin, 0.25 mg/L amphotericin B.
After one day post-confluence cells were trypsinized (Trypsin-EDTA 0.05%-0.02%), diluted (usually 1:3) and then seeded again in a new plastic flask. Each trypsinization and successive dilution in a new flask was considered as a cell passage. The parental cell line from a single flask was cultured for 50 passages following the experimental procedure described and represented in a schematic diagram in Figure 1. The medium was changed twice a week (Ferraretto et al. 2007).
Cultures, kept at 37°C in a 5% CO₂- 95% air atmosphere, were periodically checked for the presence of mycoplasma and were found to be free of contamination.
METHOD OF DIFFERENTIATION OF THE CELL LINE CACO2

SEEDING CELLS

GROWING CELLS

100% CONFLUENCY

1 DAY POST-CONFLUENCY

TRYPSIN-EDTA (0.05%-0.02%)

SUBCULTURE WITH APPROPRIATE DILUTION FACTOR

Every 2 days medium changed

Figure 1. Schematic diagram of the standardized Caco2 cell culture methodology used (Ferraretto et al. 2007).
RNA INTERFERENCE

RNA interference technique has been used to assess whether the channel TRPV6 was involved in the CPP biological effect. Sequences of siRNA for inhibiting expression of TRPV6 gene were designed according to (Irnaten, et al. 2008).

Transfection of siRNA by lipofectamine

The lipofectamine are a class of molecules characterized by a cationic portion that bind to nucleic acids and by a lipid portion which facilitates the passage of complex RNA-lipofectamine through the cell membrane. Cells (19x10^4 cells/well) were plated in 35 mm Petri dish in order to reach a 30% of confluency 24 h later. Then they were washed twice with medium without antibiotic and incubated in the same medium.

siRNA were prepared as follows: siRNA and lipofectamine (Invitrogen, Carlsbad, California) were separately diluted in medium without antibiotic and FBS and incubated for 5 minutes at room temperature. Successively the two mixtures were mixed and left at room temperature for 20 minutes in order to allow the formation of complex siRNA-Lipofectamine. After the complex formation siRNA-Lipofectamine were added in each well. 24 h later the medium with siRNA-Lipofectamine was removed and complete medium were added. 48 h the after incubation with siRNA the cells were collected for the evaluation of the silencing of gene expression.

GENE EXPRESSION ANALYSIS

Gene expression of siRNA TRPV6 was evaluated in Caco2 cells. Total RNA was isolated and purified from cell lysates using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). During RNA purification, the RNase-Free DNase Set (Qiagen) was used for residual genomic DNA digestion and the RNA isolated was quantified by RiboGreen® RNA quantitation assay (Invitrogen, Carlsbad, California). 800 ng of the isolated RNA was reverse-transcribed to cDNA employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California). 20 ng of total RNA was used as template for real-time PCR performed using the iCycler thermal cycler (Bio-Rad Laboratories). PCR mixture included 0.2 µM primers, 50 mM KCl, 20 mM Tris/HCl, pH 8.4, 0.8 mM dNTPs, 0.7 U iTaq DNA Polymerase, 3 mM MgCl2, and SYBR Green (iQ SYBR Green Supermix; Bio-Rad Laboratories) in a final volume of 20 µL. The sequences of the forward and reverse primers are shown in Table 1.

Amplification and real-time data acquisition were performed using the following cycle conditions: initial denaturation at 95 °C for 30 s, followed by 45 cycles of 10 s at 95 °C
and 30 s at 58 °C. The fold change in expression of the different genes in control and treated cells was normalized to the expression of GAPDH gene.

The specificity of the PCR product was monitored in every PCR assay by the analysis of the melting curves.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Forward TRPV6</td>
<td>5’ TACCCATGTCCCTTTGCACTC 3’</td>
<td>20</td>
</tr>
<tr>
<td>Primer Reverse TRPV6</td>
<td>5’ CATCAGGTCGCCAAAAATC 3’</td>
<td>19</td>
</tr>
<tr>
<td>Primer Forward GAPDH</td>
<td>5’ ACGGATTTGGTCTGTATGG 3’</td>
<td>19</td>
</tr>
<tr>
<td>Primer Reverse GAPDH</td>
<td>5’ CATGGGTGGAATCATATTGG 3’</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 1.** Sequences of primers for amplification of the TRPV6 gene and GAPDH.

**Western blot**

Detection of TRPV6 in Caco2 and HT-29 cells was carried out using standard immunoblotting protocol and commercially available antibodies. Cells were gently washed three times with cold PBS and then scraped into homogenization buffer containing in (mM): 20 Tris-HCl pH 7.6, 250 NaCl, 3 EGTA, 3 EDTA, protease inhibitors and 2 dithiothreitol. The crude cell lysate was homogenized, sonicated and subsequently centrifuged for 15 min at 14000 rpm. Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, UK). Equal amounts of extracted total proteins were separated on an 8% SDS-PAGE gels and transferred to PVDF membranes using standard semi-dry technique. Membranes were blocked with 5% non-fat dry milk in Tris Buffered Saline with Tween-20 (TBS-T) overnight at 4°C in presence of rabbit anti-human TRPV6 primary antibody (Santa Cruz Biotechnology, USA) used at a dilution of 1:500. After washing, membranes were incubated with HRP-rabbit secondary antibody (1:10000; Santa Cruz Biotechnology, USA) for 1 hour. Antibody protein complexes were visualized using an enhanced chemiluminescent detection kit (Pierce Biotechnology, Inc., IL). Anti-β-actin monoclonal antibody (1:20000; Sigma, USA) was used as a loading control.
MEASUREMENT OF INTRACELLULAR FREE CALCIUM CONCENTRATION AT SINGLE CELL LEVEL

As a result of TRPV6 gene silencing, the effect of CPP administration was evaluated by monitoring the cytoplasmic calcium levels in Caco2 cells in presence of 2 mM [Ca^{2+}].

Cells were loaded with 2.5 µM Fura-2 AM and 2.5 µM Pluronic F-127 in Krebs Ringer Hepes solution (KRH SD) according to the procedure already described (Cosentino et al. 2010b; Ferraretto, et al. 2001).

For the analysis of intracellular free calcium, [Ca^{2+}], see the same paragraph in Materials and Methods 1.

EVALUATION OF CELL PROLIFERATION AND APOPTOSIS

Undifferentiated and differentiated Caco2 cells were treated for 24 h with either 1280 µM CPP or 500 µM EGTA in foetal bovine serum free media, in the presence of 2 and 6 mM CaCl_2. The effect of 400 nM Bay-K8644, 30 µM Nimodipine, 30 µM Nifedipine in the presence of 2 mM CaCl_2 was determined following the same procedure. Cell proliferation was evaluated through the measurement of the bromo-deoxyuridine (BrdU) cell incorporation. Apoptosis was evaluated through the Apo-ONE® assay and Dapi staining. The procedures of the experiments were the same used for HT-29 cells and can be found in the appropriate sections in Materials and Methods 1.

STATISTICAL ANALYSIS

To determine statistically significant differences (p value<0.05 or p value<0.01), the results presented were analyzed by Student’s t-test independent two population t test, χ^2 test and ANOVA. The program used was SPSS 19 (IBM Inc.).
4.2 Results 2
EVALUATION OF THE EFFECT OF L-TYPE CALCIUM CHANNEL AGONIST BAY-K8644

When analyzed by computerized video microscopy, only 5% of Caco2 cells showed a change in cytosolic calcium concentration in response to treatment with Bay-K 8644 (Figure 2). Therefore it can be concluded that the majority of Caco2 cells is not sensitive to treatment with Bay-K 8644.

This result confirms the previous observations of a lack of expression of genes for L-type calcium channels and the consequent absence of functional channels in these cells (Nakkrasae, et al. 2010).

Figure 2. Changes in cytosolic calcium concentration in Caco2 cells after administration of 800 nM Bay K-8644. Each line in the graph represents the behavior of individual cells belonging to the cellular field analyzed. The figure represents a typical trace representative of at least three similar experiments.
EFFECT OF ACTIVATORS/ INHIBITORS OF L-TYPE CALCIUM CHANNELS ON CELL PROLIFERATION

In the presence of physiological concentrations of extracellular calcium in the medium (2 mM), neither the activator Bay-K 8644 (103.8 ± 9.4% vs control) nor the inhibitors Nimodipine and Nifedipine (76.2 ± 10% and 92.7 ± 13.6% vs control, respectively), are able to affect the proliferation rate of Caco2 cells (Figure 3).

The absence of active L-type calcium channels, dihydropyridine sensitive, in Caco2 cells leads to suppose a different interaction between the CPP and the plasma membrane in these cells.

Figure 3. Assessment of proliferation using bromodeoxyuridine assay in differentiated Caco2 cells in presence of activator/ inhibitors of L-type Ca\(^{2+}\) channels in 2mM [Ca\(^{2+}\)]\(_o\).

![Graph showing proliferation rates](image-url)
TRPV6 GENE EXPRESSION AFTER TREATMENT WITH siRNA SCRAMBLE AND siRNA TRPV6

The efficiency of TRPV6 gene silencing was analyzed by evaluating the expression of the transcript compared to treatment with siRNA scramble. In differentiated Caco2 cells the treatment with siRNA TRPV6 reduced the mRNA expression of approximately 60% compared to siRNA scramble (Figure 4).

![Figure 4](image)

**Figure 4.** Levels of TRPV6 mRNA expression in differentiated Caco2 cells following treatment with siRNA scramble and siRNA TRPV6.

The reduction in the intensity of the specific band for the protein TRPV6, between 75 and 100 kDa, in differentiated Caco2 cells treated with siRNA TRPV6 confirms the silencing of the gene coding for the channel TRPV6 confirm the silencing of the gene coding for the channel TRPV6 (Figure 5).

![Figure 5](image)

**Figure 5.** Western blot of differentiated Caco2 cells treated with siRNA scramble and siRNA TRPV6.
EVALUATION OF INTRACELLULAR CALCIUM CONCENTRATION IN siRNA TRPV6 CACO2 CELLS AFTER CPP ADMINISTRATION

Using computerized video microscopy, the cell responsiveness to CPPs as well as cytoplasmic calcium levels, were assessed in siRNA TRPV6 Caco2 cells. Regarding responsiveness, it can be seen that the percentage of cells responsive to the administration of CPP in siRNA TRPV6 cells (10.42%) is statistically lower ($\chi^2$ test p <0.01), compared to control cells (32.80%) and to siRNA scramble cells (23.90%). The responsiveness of siRNA scramble cells was not statistically different from that observed in the control ($\chi^2$ test p <0.01).

The increase in intracellular calcium concentration does not change significantly (ANOVA p <0.05) between control cells and siRNA treated cells (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>% Responsive cells</th>
<th>$\Delta [\text{Ca}^{2+}]$ nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.80</td>
<td>$28.67 \pm 7.87^a$</td>
</tr>
<tr>
<td>siRNA scramble</td>
<td>23.90</td>
<td>$34.68 \pm 11.73^a$</td>
</tr>
<tr>
<td>siRNA TRPV6</td>
<td>10.42$^z$</td>
<td>$27.68 \pm 5.69^a$</td>
</tr>
</tbody>
</table>

**Table 2.** Analysis of the CPP effect on intracellular calcium concentration as determined by computerized videomicroscopy in control cells, siRNA scramble cells and SiRNA TRPV6 cells. The statistical significance for cell responsiveness was assessed using the $\chi^2$ test ($^z$ significantly different from control, p <0.01), while the variations in the intracellular calcium concentration were analyzed by ANOVA (values in the same column with the same letter are not significantly different, p <0.05).

Next figures show the trend of the changes in the cytosolic calcium after CPP administration in control cells, in siRNA scramble cells and siRNA TRPV6 cells. Experiments were performed using computerized video microscopy.
Figure 6. Changes in cytosolic calcium in control cells (A), siRNA scramble cells (B) and siRNA TRPV6 cells (C), after administration of 1280 µM CPPs. Each line represents a single cell behavior taken from the chosen cellular field. Each graph is representative of at least three independent experiments.
EVALUATION OF INTRACELLULAR CALCIUM CONCENTRATION IN siRNA TRPV6 HT-29 CELLS AFTER CPP ADMINISTRATION

For comparison, also in HT-29 cells was silenced the gene TRPV6 (60% decrease of expression similar to that obtained in Caco2 cells), and analyzed the cellular responsiveness to CPPs, with a cellular response by 67%, identical to the value observed in the control (67%), in agreement with previous studies (Figure 7) (Munaron, et al. 2004).

Figure 7. Changes in cytosolic calcium in siRNA TRPV6 HT-29 cells after administration of 1280 µM CPP. Each line represents a single cell behavior taken from to the chosen cellular field. The graph is representative of at least three independent experiments.
EFFECT OF CPP ON PROLIFERATION RATE IN UNDIFFERENTIATED/DIFFERENTIATED CACO2 CELLS

In undifferentiated cells, both under physiological conditions and in the calcium overload, the administration of CPP did not cause any statistically significant change (86.12 ± 11.08% vs. control for 2 mM [Ca$^{2+}$]o and 72.21 ± 17.07% vs. control for 6 mM [Ca$^{2+}$]o).

Even EGTA did not cause any statistically significant change (89.32% ± 8.45 vs. control for 2 mM [Ca$^{2+}$]o and 63.53% ± 18.17 vs. control for [Ca$^{2+}$]o 6 mM) (Figure 8A).

In differentiated cells, the CPP treatment (100.33 ± 2.08% vs. control for 2 mM [Ca$^{2+}$]o and 88.67% ± 11.59 vs. control for 6 mM [Ca$^{2+}$]o) and EGTA treatment (96.70 ± 1.50% vs. control for 2 mM [Ca$^{2+}$]o and 81.33 ± 12.86% vs. control for 6 mM [Ca$^{2+}$]o) do not cause any statistically significant changes in proliferative rate, either in physiological or in calcium overload (Figure 8B).

Figure 8. Assessment of proliferation using bromodeoxyuridine assay in undifferentiated (A) and differentiated (B) Caco2 cells, after administration of 1280 µM CPP or 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments.
EFFECT OF CPP ON APOPTOSIS IN UNDIFFERENTIATED/DIFFERENTIATED CACO2 CELLS

The evaluation of apoptosis by Apo-One assay showed that in undifferentiated cells, in the presence of both calcium concentrations, the administration of CPPs led to a statistically significant increase in apoptosis (Figure 9). These results were also confirmed by DAPI staining (Figure 10A). Conversely, in differentiated cells (Figure 8B) CPPs have no effect. The EGTA administration induces a statistically significant increase of apoptosis in undifferentiated cells at 2 mM [Ca\textsuperscript{2+}]\textsubscript{o}, and in differentiated cells both at 2 mM [Ca\textsuperscript{2+}]\textsubscript{o} and 6 mM [Ca\textsuperscript{2+}]\textsubscript{o}.

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}]\textsubscript{o}</th>
<th>Undiff. Caco2 (% vs Crt)</th>
<th>Diff. Caco2 (% vs Crt)</th>
<th>Undiff. Caco2 (% vs Crt)</th>
<th>Diff. Caco2 (% vs Crt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>CPP</td>
<td>131.67 ± 7.33*</td>
<td>120.06 ± 19.56</td>
<td>163.01 ± 17.20*</td>
</tr>
<tr>
<td></td>
<td>EGTA</td>
<td>113.12 ± 9.50</td>
<td>97.33 ± 15.29</td>
<td>93.13 ± 9.34</td>
</tr>
<tr>
<td>6 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

Table 3. Values of apoptosis obtained by Apo-one assay in undifferentiated and differentiated Caco2 cells (* significantly different from control, Student- t test, p <0.05).

Figure 9. Assessment of apoptosis by Apo-One assay in Caco2 cells undifferentiated (A) and differentiated (B) following administration of 1280 µM CPP and 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments, (* significantly different from control, Student- t test, p <0.05)
Table 4. Apoptosis rates obtained by DAPI staining in undifferentiated and differentiated Caco2 cells (* significantly different from control, Student-t test, p <0.05).

<table>
<thead>
<tr>
<th></th>
<th>[Ca^{2+}]_0 2 mM</th>
<th>[Ca^{2+}]_0 6 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiff. Caco2 (% vs Crt)</td>
<td>Diff. Caco2 (% vs Crt)</td>
</tr>
<tr>
<td>CPP</td>
<td>150.91 ± 11.2*</td>
<td>95.33 ± 1.40</td>
</tr>
<tr>
<td>EGTA</td>
<td>131.06 ± 13.94*</td>
<td>149.89 ± 2.66*</td>
</tr>
</tbody>
</table>

Figure 10. Evaluation of the apoptosis rate by DAPI staining in undifferentiated (A) and differentiated (B) Caco2 cells following administration of 1280 µM CPP or 500 µM EGTA. Each bar represents the mean ± S.D. of 3 analogous experiments, (* significantly different from control, Student-t test, p <0.05).
4.3 Conclusions 2
In recent years it has been established that an increase in the consumption of calcium and vitamin D is able to prevent the onset of colorectal cancer (van den Brandt and Goldbohm 2006).

The complex mechanisms that regulate the development of a differentiated or a tumor phenotype in the colon are closely associated with molecular mechanisms dependent on β-catenin and CaSR (Haegebarth and Clevers 2009; MacLeod, et al. 2007; Whitfield 2009), although it seems that the target of a diet high in calcium is the TRPV6 channel. In human colon this channel is associated with early stages of carcinogenesis.

A high amount of calcium in the diet seems to induce in a healthy intestinal epithelium an inhibition of cellular growth and a decreased expression of TRPV6, while vitamin D in high doses induces over-expression of TRPV6 leading to tumor promotion, effectively neutralizing the effect of a calcium rich diet (van den Brandt and Goldbohm 2006).

When other nutrients are considered, the situation becomes more complicated. In fact, calcium and vitamin D are contained in milk and dairy products, known to be good sources.

Based on the results found in HT-29 cells, the purpose of this study was to clarify whether the biological effect of CPPs in Caco2 cell line was also due to modulation of L-type calcium channels, or was due to an interaction with the channel TRPV6, the main responsible for the transcellular calcium transport in the intestinal epithelium. In fact Caco2 cell line when differentiated represent the most widely used model in studies of transport of xenobiotics and nutrients because it is comprised mainly of absorptive enterocytes.

In contrast, the HT-29 cell line, when differentiated, retains the characteristics of an heterogeneous population and may include, in addition to absorbing cells, mucus goblet as well as entero-endocrine cells (Hekmati, et al. 1990). Moreover, the assumption of an interaction of CPPs with different molecular structures at the plasma membrane of HT-29 cells compared to Caco2, is supported by the different shapes and extent of response to the CPPs by the two cell lines (Cosentino et al. 2010b).

Calcium uptake experiments conducted in differentiated Caco2 cells in the presence of a selective agonist of L-type calcium channel, unlike the HT-29 cells, showed significant changes in the intracellular calcium concentration in only 5% of total analyzed cells.

This result confirms the lack of expression of genes for L-type calcium channels sensitive to dihydropyridines and the consequent absence of functional channels in these cells (Nakkrasae et al. 2010; Thongon, et al. 2009). To confirm this result, the
proliferation of differentiated Caco2 cells is not modulated by the same agonist/antagonists of L-type calcium-channel.

The absence of the active L-type calcium channels in Caco2 cells leads to assume a different interaction between CPPs and the plasma membrane in these cells. Among the various channels present and involved in the influx of calcium into the intestinal epithelium, the most important is TRPV6 which is constitutively expressed in Caco2 cells (Peleg et al. 2010).

Since there are no agonists or antagonists for this channel, to verify a possible interaction with CPP experiments were conducted on cells silenced for the TRPV6 gene. The 60% reduction of mRNA expression and the absence of specific band in Western blot experiment in Caco2 cells treated with siRNA TRPV6 has confirmed the silencing of the gene coding for the channel.

The computerized video microscopy experiments have shown that in siRNA TRPV6 cells there is a statistically significant decrease in the percentage of responsiveness to CPPs.

Further confirmation of the interaction of CPPs with TRPV6 in Caco2 cells, comes from the silencing of the same channel in HT-29 cells. The results show that in these cells the responsiveness to CPPs is the same between control and siRNA (67%), indicating that in these cells the interaction of CPPs involves other channels, particularly L-type.

Based on these preliminary results, therefore we can assume the voltage-gated L-type calcium channel and TRPV6 as the main channels involved in calcium uptake induced by CPPs in HT-29 and Caco2 cells respectively, but certainly not the only channels involved.

The proliferative activity in Caco2 cells does not appear to be influenced by treatment with CPPs, while the apoptotic activity is subject to an increase in undifferentiated cells and a reduction in differentiated ones in both physiological and overload extracellular calcium concentrations.

These results do suggest that the interaction of CPP with the channel TRPV6 modulates cell viability in Caco2 cells, similarly to what happens in HT-29 cells with the L-type calcium channels. Moreover this result confirms, even in Caco2 cells, the ability of CPPs, as calcium ion chelators and activators of the mineral uptake in differentiated cells, to influence the cellular mechanisms underlying the development and/or regression of the tumor phenotype.

In addition, experiments are in progress in our laboratory to evaluate the proliferative and apoptotic rate of Caco2 cells after the silencing of TRPV6 channel, and the biological role exerted by CPPs.
Therefore, *in vitro*, CPPs are able to modulate the absorption of calcium into Caco2 cells through the transcellular route by interacting with the channel TRPV6. In addition to the channel encoded by the gene TRPV6, CPPs may interact with other receptors and/or channels in the membrane. One of these receptors may be the CaSR, a membrane calcium sensor, which is able to sense changes in \([\text{Ca}^{2+}]_{o}\) and coupled them to changes in \([\text{Ca}^{2+}]_{i}\) (Hoenderop, et al. 2005). In fact, in adult intestinal cells, the expression of CaSR regulates the entry of calcium ions and activates a series of mechanisms that lead the cell to stop proliferation, to differentiate and finally undergo apoptosis. In the case of tumorigenesis, such as polyps and adenomas, there is a deregulation of these mechanisms, whereby an increase in extracellular calcium concentration leads to higher rates of uncontrolled cell replication, increasing tumor size (Whitfield 2009).

However these *in vitro* assessments need support and validation in vivo, where the modulating action of these peptides may be easily affected even by the physiological conditions of the individual (such as the pH of the gastrointestinal tract) as well as by the composition of the meal.

Ion channels are involved in cell homeostasis and their influence in cell behavior is of fundamental importance during development and tumor progression. Defects in the channels are called channelopathies and are the basis of many diseases, it is therefore essential to study the molecular mechanisms by which these channels are involved in biological processes, and the interaction with nutrients and/or molecules in foods that constitute our diet.

There is no doubt that CPPs are a key resource, whether considered as potential adjuvants in the process of intestinal calcium absorption and as promoters of numerous cellular functions through the activation of calcium signaling pathways, with all the implications thereof.

The role of such peptides as "functional foods" or nutraceuticals for the intestinal epithelium may therefore represent an important opportunity for the nutraceutical industry, which currently represents a growing market (Vergari, et al. 2011).
4.4 REFERENCES 2


Irnaten M, Blanchard-Gutton N & Harvey BJ 2008 Rapid effects of 17beta-estradiol on epithelial TRPV6 Ca2+ channel in human T84 colonic cells. *Cell Calcium* 44 441-452.

MacLeod RJ, Hayes M & Pacheco I 2007 Wnt5a secretion stimulated by the extracellular calcium-sensing receptor inhibits defective Wnt signaling in colon cancer cells. *Am J Physiol Gastrointest Liver Physiol* **293** G403-411.


Wood RJ, Tchack L & Taparia S 2001 1,25-Dihydroxyvitamin D3 increases the expression of the CaT1 epithelial calcium channel in the Caco-2 human intestinal cell line. *BMC Physiol* **1** 11.
5. Appendix
Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells

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Bone

\textbf{Abstract}

Casein phosphopeptides (CPPs), originating by in vitro and/or in vivo casein digestion, are characterized by the ability to complex and solubilize calcium ions preventing their precipitation. Previous works demonstrated that CPPs improve calcium uptake by human differentiated intestinal tumor cell lines, are able to re-mineralize carious lesions in a dental enamel, and, as components of a diet, affect bone weight and calcium content in rats. The aim of the present study was to evaluate if CPPs can directly modulate bone cells activity and mineralization. Primary human osteoblast-like cells were established in culture from trabecular bone samples obtained from waste materials during orthopedic surgery. Commercial mixtures of bovine casein phosphopeptides were used. The CPP dependent intracellular calcium rises were monitored at the single cell level through furaz-fluorescence assays. Results show that CPPs: (i) stimulate calcium uptake by primary human osteoblast-like cells; (ii) increase the expression and activity of alkaline phosphatase, a marker of human osteoblast differentiation; (iii) affect the cell proliferation rate and the apoptotic level; (iv) enhance nodule formation by human SaOS-2. Taken together these results confirm the possibility that CPPs play a role as modulator of bone cell activity, probably sustained by their ability as calcium carriers. Although the exact mechanism by which CPPs act remains not completely clarified, they can be considered as potential anabolic factors for bone tissue engineering.

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Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with voltage-operated L-type calcium channels

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Abstract

At the intestinal level, proliferation and apoptosis are modulated by the extracellular calcium concentration; thus, dietary calcium may exert a chemoprotective role on normal differentiated intestinal cells, while it may behave as a carcinogenesis promoter in transformed cells. Calcium in milk is associated with casein and casein phosphopeptides (CPPs), hence is preserved from precipitation. CPPs were demonstrated to induce uptake of extracellular calcium ions by in vitro intestinal tumor HT-29 cells but only upon differentiation. Here, the hypothesis that CPPs could differently affect proliferation and apoptosis in undifferentiated and differentiated HT-29 cells through their binding with calcium ions was investigated. Results showed that CPPs protect differentiated intestinal cells from calcium overload toxicity and prevent their apoptosis favoring proliferation while inducing apoptosis in undifferentiated tumor cells. The CPP effect on undifferentiated HT-29 cells, similar to that exerted by ethyleneglycol-O,O′-bis(2- aminoethyl)−N,N′,N′′-tetraacetic acid (EGTA), is presumably due to the ability in binding the extracellular calcium. The effect on differentiated HT-29 cells is coupled to the interaction of CPPs with the voltage-operated L-type calcium channels, known to activate calcium entry into the cells under depolarization and to exert a mitogenic effect; the use of an agonist potentiates the cell response to CPPs, while the antagonists abolish the response to CPPs (96% of examined cells) or reduce both the percentage of responsive cells and the increase of intracellular calcium concentration. Taken together, these results confirm the potentialities of CPPs as nutraceuticals/functional food and also as modulators of cellular processes connected to the expression of a cancer phenotype.

Keywords: Casein phosphopeptides; Intestinal cells; Calcium; Depolarization; Calcium channels; Cell cycle

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ABSTRACT
The gastrointestinal digestion of casein gives rise to a series of peptides displaying particular features and biological activity, such as the immunostimulant, ACE inhibitory, antihypertensive, opioid agonist/antagonist, antithrombotic and antimicrobial peptides. A last group of peptides, named casein phosphopeptides, are defined mineral carriers and originate from the proteolytic cleavage of αs1-, αs2- and β-casein. These casein phosphopeptides are characterized by the presence of phosphate linked to serine residues all over the amino acid sequence but, above all, localized in a particular Ser(P)-Ser(P)-Ser(P)-Glu-Glu sequence, known as the “acidic motif”. Although casein-derived phosphopeptides (CPPs) correspond to different phosphorylated regions of αs1-, αs2- and β-caseins, all CPPs present the “acidic motif”, which was highly conserved among species and centuries. The presence of phosphate confer them a high negative charge responsible for their further proteolysis and undiscussed ability to bind minerals such as calcium, iron and zinc. Numerous studies aimed to demonstrate the possibility to use the CPP mineral binding capacity for improving the absorption of calcium and iron at intestinal level, as well as for enhancing remineralization of tooth enamel. Besides the controversial results obtained with calcium transport studies performed using rat intestinal preparations, the use of in vitro human intestinal cell models do confirm that CPPs induce calcium uptake in differentiated intestinal cells and this action is correlated to their ability to form aggregates of defined size with calcium ions. Also in human primary osteoblast like cells CPPs increase the intracellular calcium concentration and the in vitro cell mineralization. In all these events the calcium bound to CPPs represents the fraction of soluble mineral involved in the process. Finally, new and interesting potentialities for CPPs come from recent data displaying a modulation of the intestinal immune system by triggering cytokine secretion and stimulating IgA production, the release of IL-6 cytokine in human epithelial intestinal cell lines, the modulation of cell viability, i.e. proliferation and apoptosis in different human cell cultures. Taken together these findings claimed for a real possibility to use CPPs as functional food, due to their presence in dairy products and their physiological formation in vivo, and for nutraceuticals, due to possibility to add to mouth rinse solutions, toothpaste and
chewing gum, but also to confectionary products (breakfast foods, sweets, cakes, ice-cream, milk, powdered milk, yogurt, cheese, sport drinks, mayonnaise..) due to their good solubility in water, which will allow to use these peptides as an easy-to-formulate ingredient in food.
EVALUATION OF A POSSIBLE DIRECT EFFECT BY CASEIN PHOSPHOPEPTIDES ON PARACELLULAR AND VITAMIN D CONTROLLED TRANSCELLULAR CALCIUM TRANSPORT MECHANISMS IN INTESTINAL HUMAN HT-29 AND CACO2 CELL LINES


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Abstract

Intestinal cells are continuously exposed to food whose components are able to modulate some of their physiological functions. Among the bioactive food derivatives are casein phosphopeptides (CPPs), coming from the in vitro or in vivo casein digestion, which display the ability to form aggregates with calcium ions and to increase the uptake of the mineral in differentiated intestinal human in vitro HT-29 and Caco2 cells. The present study aims to determine a possible modulation by CPPs of the paracellular and/or transcellular calcium absorption in these two cell lines. The paracellular calcium transport was determined by TEER measurements in Caco2 cells and by Lucifer Yellow flow in HT-29 cells. The possible modulation of transcellular calcium absorption by CPPs was investigated by determining the mRNA expression for TRPV6 calcium channel and VDR receptor in 1,25(OH)2D3 treated undifferentiated/differentiated cells. Results obtained point out that: i) CPPs do not affect paracellular calcium absorption; ii) 1,25(OH)2D3 increases the TRPV6 mRNA expression in both types of cells; iii) CPPs per se are not able to affect the VDR and TRPV6 mRNA expression; iv) TRPV6 calcium channel mRNA expression slightly increases in 1,25(OH)2D3 treated undifferentiated HT-29 cells and in undifferentiated/differentiated Caco2 cells after CPP administration, though this increase is not statistically significant. This slight effect of CPPs could be mainly ascribed to their ability to bind extracellular calcium, a known modulator of the inactivation of the TRPV6 channel. Since proliferation and apoptosis in tumor cells like undifferentiated cells are modulated by calcium and the TRPV6 channel seems to be involved in the cancer progression, the results here presented are to be considered indicative of a possible influence by food factors like CPPs on physiological processes in the intestinal tumor cells.
CASEINPHOSPHOPEPTIDES AS BIO MODULATORS OF INTESTINAL CELL FUNCTIONS RELATED TO NORMAL/TUMOR PHENOTYPES

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Keywords: intestinal cells, caseinphosphopeptides, calcium, tumor

Introduction: It is well known that dietary calcium exerts a chemoprotective role on normal differentiated intestinal cells, while it behaves as a carcinogenesis promoter on aberrant colonocytes and adenomatous crypts. These different functions can be achieved through the modulation of proliferation and/or differentiation processes. Milk represents the major source of calcium in the diet, due to the high availability of the mineral bound to casein and caseinphosphopeptides (CPPs). CPPs, derived by in vitro or in vivo casein hydrolysis, are able to induce calcium uptake in human intestinal tumor cells differentiated in vitro toward an enterocycte phenotype.

Objectives: The CPP properties and abilities could be correlated with a possible activation of pathways of calcium signalling in intestinal cells either in standard growth condition, or in calcium overload as it might occurs following a meal.

Design: Undifferentiated and differentiated human intestinal cell lines HT-29 and Caco2, as in vitro cellular models of intestinal epithelium, were assayed for: cell proliferation (bromodeoxyuridine incorporation); apoptosis (caspase 3/7 activation, DAPI staining); intracellular calcium increases (Video-imaging experiments using Fura2).

Results: In HT-29 cells, CPPs differently affected proliferation rate and apoptosis in undifferentiated toward differentiated cells, acting through the modulation of the voltage operated L-type calcium channels, known to activate calcium entry into the cells under depolarization and to exert a mitogenic effect. In Caco2 cells, CPPs did not significantly affect proliferation and apoptosis, moreover they induced calcium uptakes mainly through the TRPV6 channel, known as the epithelial channel responsible for the calcium absorption at duodenum level.

Conclusion: Taken together these results demonstrate the ability for CPPs, through the binding with calcium ions and the stimulated ingressio of these ions in differentiated cells, to modulate biological activity strictly related to normal or cancer phenotype, thus opening the way for a use of CPPs as nutraceutical/functional food.
Milk and dairy products are known sources of bioavailable calcium for its association with casein, whose proteolysis produces caseinphosphopeptides (CPPs). CPPs are phosphorylated peptides able to bind and solubilise calcium. In human intestinal tumor cells differentiated in vitro toward an enterocytc phenotype, they also induce a calcium uptake. Moreover, in human in vitro osteoblasts, CPPs favour the mineralization of the extracellular matrix. CPPs can differently affect proliferation and apoptosis in differentiated and/or tumor intestinal cells. Due to all these properties, CPPs may be considered as potential nutraceutical/functional food.
CASEIN PHOSPHOPEPTIDES: FROM MINERAL CARRIERS TO MODULATORS OF BIOLOGICAL ACTIVITY IN INTESTINAL CELLS

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Aims. Calcium introduced with the diet and absorbed in the intestinal lumen in addition to the known functions in bone and teeth and the maintenance of plasma calcium concentration (calcemia), is able to modulate biological activities of intestinal cells such as proliferation, apoptosis and differentiation, involved in the development and/or regression of cancer. Calcium in milk is associated with casein and casein phosphopeptides (CPPs). CPPs, derived by in vitro or in vivo casein hydrolysis, are known to bind calcium and induce calcium uptake in human intestinal tumor cells differentiated in vitro toward an enterocytic phenotype. These CPP properties and abilities could be correlated with a possible activation of pathways of calcium signalling in intestinal cells either in standard growth condition, or in calcium overload as it might occurs following a meal.

Materials and Methods. Undifferentiated and differentiated human intestinal cell line HT-29 was used as in vitro cellular model of tumor or physiologic intestinal epithelium. Cell proliferation rate was estimated by incorporation of bromodeoxyuridine. Cell apoptosis was measured by caspase 3/7 activation and nuclei DAPI staining. Intracellular calcium concentration was quantified by Video-imaging experiments using Fura2.

Results. In undifferentiated HT-29 DMEM cells, that partially expresses the CaSR and mimics the behavior of tumor cells in their early stages, CPPs increase apoptosis, both at physiological calcium concentration and in calcium overload. These results are also shared by the calcium chelator EGTA, and are presumably due to the ability in binding the extracellular calcium. In differentiated RPMI HT-29 cells, CPPs: i) increase proliferation, especially in calcium overload; ii) do not affect apoptosis; iii) protect from calcium overload toxicity. On the contrary, EGTA behaves as a cytotoxic agent, decreasing proliferation and increasing apoptosis. The CPP effect in differentiated RPMI cells is related to their ability of activating cell calcium entry, through the interaction with the voltage operated L-type calcium channels, known to activate calcium entry into the cells under depolarization and to exert a mitogenic effect. Moreover, CPPs displays a depolarizing effect on cell membrane.

Conclusion. Taken together these results demonstrate the ability for CPPs, through the binding with calcium ions and the stimulated ingression of these ions in differentiated cells, to modulate biological activity strictly related to normal or cancer phenotype thus opening the way for a use of CPPs as nutraceutical/functional food.
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I CASEINOFOSFOPEPTIDI: BIO-MODULATORI DELLE ATTIVITÀ BIOLOGICHE DELLE CELLULE INTESTINALI E DEI CANALI DEPUTATI ALL’ASSORBIMENTO DEL CALCIO

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Premesse. Il calcio introdotto con la dieta e presente nel lume intestinale oltre ad essere assorbito e concorrere alla stabilizzazione della calcemia è in grado di modulare attività biologiche delle cellule intestinali come proliferazione, apoptosi e differenziamento, coinvolte nello sviluppo e/o regressione di patologie quali tumore, infiammazione cronica e malattie autoimmuni. La trasduzione del segnale dall’ambiente extracellulare all’interno delle cellule è operata dal Calcium sensing receptor (CaSR), recettore accoppiato a G-proteine, in grado di sentire variazioni della concentrazione di calcio extracellulare e di attivare una complessa via di segnalazione. La forma attiva della vitamina D, l’1,25(OH)2D3, regola l’espressione del CaSR e del canale epiteliale TRPV6 coinvolto nel trasporto transcellulare del calcio, tramite il legame con il suo recettore nucleare VDR.

Obiettivo. Il presente studio intende valutare se i caseinofosfopeptidi (CPP), che si originano dalla proteolisi della caseina, complessando gli ioni calcio a livello extracellulare possano influenzare le attività cellulari modulate dal legame del calcio extracellulare con il CaSR.

Metodi. L’attività proliferativa di cellule Caco2 differenziate in coltura in vitro è stata misurata mediante incorporazione di Bromo-deossi-Uridina. L’apoptosi è stata monitorata mediante attivazione delle caspasi effettrici 3 e 7. L’espressione genica del recettore VDR come pure del canale TRPV6 è stata valutata in cellule Caco2 indifferenziate e differenziate mediante RT-PCR e quantificata rispetto all’espressione del gene reporter GAPDH. I CPP utilizzati sono costituiti da una miscela commerciale comprensiva peptidi derivanti dall’alfa e beta caseina bovina.

Risultati. Il trattamento delle cellule con i CPP o con CPP e 1,25(OH)2D3 non modifica l’espressione del recettore VDR. Il trattamento di cellule Caco2 differenziate con l’1,25(OH)2D3 aumenta di circa 10 volte l’espressione del canale TRPV6. L’aggiunta di CPP in coltura a cellule differenziate e pretrattate con l’1,25(OH)2D3 aumenta significativamente l’espressione del canale TRPV6. In presenza di concentrazioni elevate di calcio extracellulare i CPP ne annullano l’attività antiproliferativa e pro-apoptotica.

Conclusioni. La capacità legante il calcio dei CPP si traduce a livello intestinale nella modulazione di eventi quali proliferazione, apoptosi ed espressione del canale per il calcio TRPV6. Questi risultati indicano la possibilità di considerare i CPP come nutraceutici a livello intestinale.
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