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Calcium-Dependent c-*myc* Proto-Oncogene Expression and Proliferation of CACO-2 Cells: A Role for a Luminal Extracellular Calcium-Sensing Receptor

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The human colonic cell line Caco-2 responds to a reduction of ambient $[Ca^{++}]_o$ to levels at and below 0.25 mM by a twofold increase in $[^3H]$thymidine labelling of their DNA. $[Ca^{++}]_o$ signals sensed preferentially across the luminal aspect of Caco-2 cells, are rapidly (4 h) transduced via PKC activation into up to sixfold increases in c-*myc* expression. This suggests the presence of a $[Ca^{++}]_o$-sensing membrane receptor (CaR) similar to that described by Brown et al. (1) in parathyroid and kidney cells. By RT-PCR we were able to amplify a 426 bp fragment from Caco-2 mRNA with 98% nucleotide identity to a part of the coding region for the extracellular domain of the parathyroid CaR. Immunohistochemical staining with a monoclonal anti-parathyroid CaR antibody demonstrates CaR protein at the plasma membrane of confluent Caco-2 cells. Our results imply that the intestinal CaR is a potential mediator for the transduction of low luminal $[Ca^{++}]_o$ into tumor promoting signals in human colonocytes

There is mounting evidence from epidemiological studies that the incidence of colorectal cancer in humans is inversely correlated with dietary calcium intake (2). This implies that adequate calcium nutrition is a key factor in preventing this malignancy. The beneficial effect of dietary calcium or calcium supplementation, respectively, has been attributed in part to the ability of the mineral ion to form insoluble salts with potentially tumorigenic bile acids (3) or to modify their relative amount in duodenal bile (4). However, extracellular calcium ($[Ca^{++}]_o$) apparently is also a direct modulator of colonocyte proliferation. In this respect we were able to demonstrate that the proliferative potential of the colonocytic cell line Caco-2 increases with decreasing levels of $[Ca^{++}]_o$ in the culture medium (5,6). Further evidence that low $[Ca^{++}]_o$ is a growth-promoting stimulus was obtained by the observation that up-regulation of c-*myc* proto-oncogene expression is a key factor in $[Ca^{++}]_o$-dependent proliferation of Caco-2 cells (7). However, the mechanism by which changes in $[Ca^{++}]_o$ could be transformed into growth promoting stimuli remained an enigma. In the present study we present evidence that the luminal plasma membrane of Caco-2 cells has the unique ability to sense variations in $[Ca^{++}]_o$ and to transduce respective signals along the PKC pathway into rapid elevation of c-*myc* expression. This in all likelihood is due to the presence of the same extracellular calcium-sensing receptor (CaR) recently cloned from parathyroid, kidney and thyroid C cells (1, 8).

METHODS AND MATERIALS

Culture media. Dulbecco's modified Eagles' medium (DMEM) was supplemented with 4.0 mM glutamine, 10% fetal calf serum (heat-inactivated at 56° C for 30 min), 20 mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin (Life Technologies, Gaithersburg, MD). Final $[Ca^{++}]_o$ in this medium was 1.80 mM. For media with lower $[Ca^{++}]$ a specially prepared DMEM (without calcium, Life Technologies) was used and calcium was substituted as appropriate.

Cell culture. Caco-2 cells were routinely seeded in 24-well Falcon plastic tissue culture dishes (15000 cells/ml), on plastic filters with 0.4 mm pore size (Falcon cell culture inserts), or on tissue culture chamber slides (Nunc Inc., Naperville, IL.) for immunocytochemistry, as appropriate. Cells were cultured in DMEM containing 10% FCS until confluency. For further culture, FCS was substituted with a solution containing 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Becton-Dickinson, Bedford, MA.). After 48 h, cells were exposed to media containing different levels of $[Ca^{++}]_o$, as indicated.

Cell proliferation assay and cell counting. DNA synthesis was assessed by measuring incorporation of $[^3H]$thymidine into cellular DNA. Cells were incubated at 37°C in DMEM containing 4 μCi/ml of $[^3H]$thymidine (specific acttivity 70 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) for 6 h.

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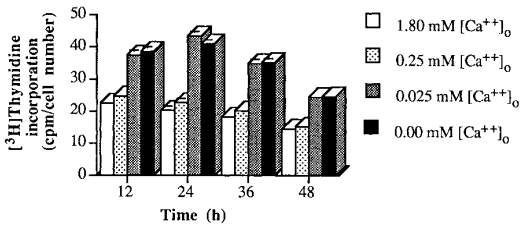


FIG. 1. Mitogenic effect of low $[Ca^{++}]_o$ on Caco-2 cells. Data are means±SEM (vertical bars, n=6) from a typical experiment (out of six).

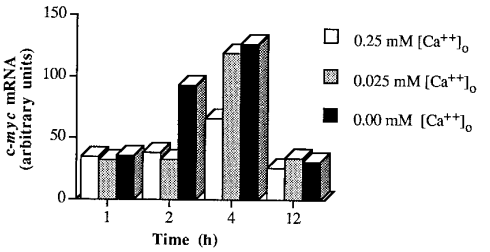


FIG. 2. Time-course of c-*myc* mRNA upregulation by exposure of Caco-2 cells to low $[Ca^{++}]_o$. Data are arbitrary densitometric units normalized to 18 S mRNA from a typical experiment (out of three).

For cell counting, trypsinized cells were suspended in a balanced electrolyte solution (Isoton II, Coulter Euro Diagnostics, Krefeld, FRG) and counted in a Coulter Counter (Model D, Coulter Electronics, Luton, GB).

RNA preparation and Northern blot analysis. Total RNA was prepared with the Trizol reagent (Life Technologies, Gaithersburg, MD). 20 μg were size-fractionated on a 0.8% agarose-formaldehyde gel and blotted onto nylon (Millipore, Bedford, MA) membranes. A 358 bp Cla1 - Msp1 fragment from exon 3 of c-*myc* was ^{32}P-labeled by random priming (Promega, Madison, WI) and used for hybridization as described before (7). For normalization, c-myc signals were compared to the 18S ribosomal RNA band.

PCR amplification. 500 ng poly(A^+) RNA was used for the synthesis of single stranded cDNA (cDNA synthesis kit, Life Technologies, Gaithersburg, MD). The resultant first-strand cDNA was used for the PCR procedure. PCR was performed at a final concentration of 1× Pfu buffer, 0.25 mM dNTP, 0.25 μM of forward primer from exon 5 of the human parathyroid CaR, 0.25 μM of the reverse primer from exon 7 and 10 U of cloned Pfu polymerase (Stratagene, La Jolla, CA). The primer sequences were: 5′-CGGGGTACCTTAAGCACCTACGGCATCTAA-3′, sense; 5′-GCTCTAGAGTTAACGCGATCCCAAAGGGCTC-3′, antisense. Intron-spanning primers were employed to avoid confusion arising from amplification of genomic DNA.

Sequencing of CaR RT-PCR products. PCR products were digested with Kpn I and Xba I and ligated into the plasmid pBluescript SK^+ (Stratagene, La Jolla, CA) for bidirectional sequencing (dideoxy chain termination method, Applied Biosystems model 373A automated sequencer, Department of Genetics, Children's Hospital, Boston, MA). Further nucleotide analyses were carried out using GeneWorks software (version 2.3.1, IntelliGenetics, Mountain View, CA).

Immunocytochemistry was carried out as described previously (9, 10) with a mouse monoclonal antibody raised against a peptide within the extracellular domain of the human parathyroid CaR (residues 214 to 236).

RESULTS AND DISCUSSION

When Caco-2 cells were grown in DMEM containing 1.80 mM $[Ca^{++}]_o$ until 2 days past confluency and then exposed to graded concentrations of low $[Ca^{++}]_o$, they exhibited a rapid mitogenic response below a threshold concentration of 0.25 mM $[Ca^{++}]_o$ as indicated by a significant increase in [^{3}H]thymidine labelling at 12 h, with a peak at 24 h.

Thymidine labeling then steadily declined but was still significantly elevated at 48 h when compared to that of cells incubated at 1.80 mM $[Ca^{++}]_o$ (Fig. 1). The mitogenic stimulus of low $[Ca^{++}]_o$ also became obvious when mRNA expression levels of c-*myc* proto-oncogene were assessed under the same experimental conditions by Northern blot analysis (Fig. 2): Reduction of $[Ca^{++}]_o$ into the 0.0-0.25 mM range led to upregulation of c-myc mRNA, which peaked at 4 h and then rapidly declined to unstimulated levels at 12 h.

Evidence that mitogenic signals resulting from variations in $[Ca^{++}]_o$ are transduced into a cellular pathway involving activation of protein kinase C (PKC) comes from the observation that after 4 h incubation with 137 μM 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7), a potent PKC inhibitor, the mitogenic response induced by exposure of Caco-2 cells to zero $[Ca^{++}]_o$ for 24 h (cf. Fig. 1) is effectively blunted (not shown).

Confluent Caco-2 cells were then grown on plastic

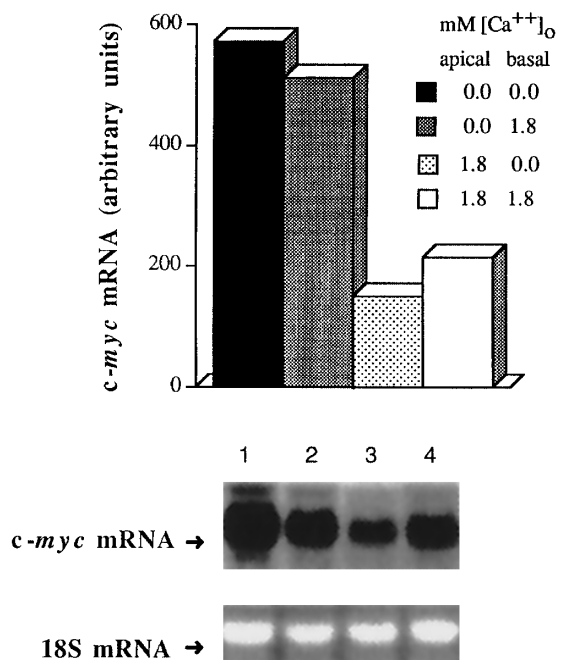
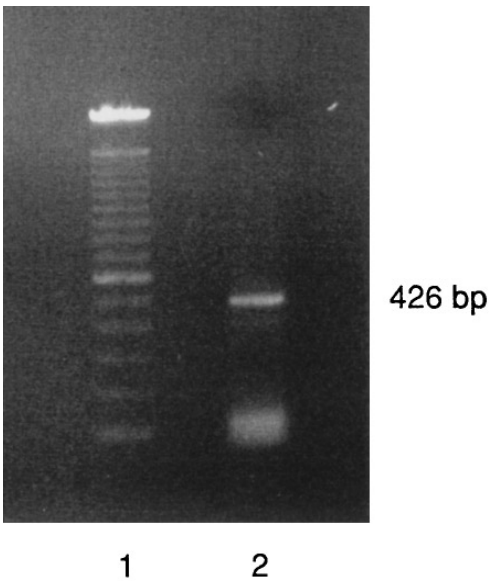


FIG. 3. Northern blot analysis of c-*myc* mRNA levels in Caco-2 cells exposed to different $[Ca^{++}]_o$ (mM): apical 0.0, basal 0.0 (lane 1); apical 0.0, basal 1.8 (lane 2); apical 1.8, basal 0.0 (lane 3); apical 1.8, basal 1.8 (lane 4). Upper part: corresponding densitometric readings for c-*myc* mRNA normalized to 18S mRNA from a typical experiment (out of three).



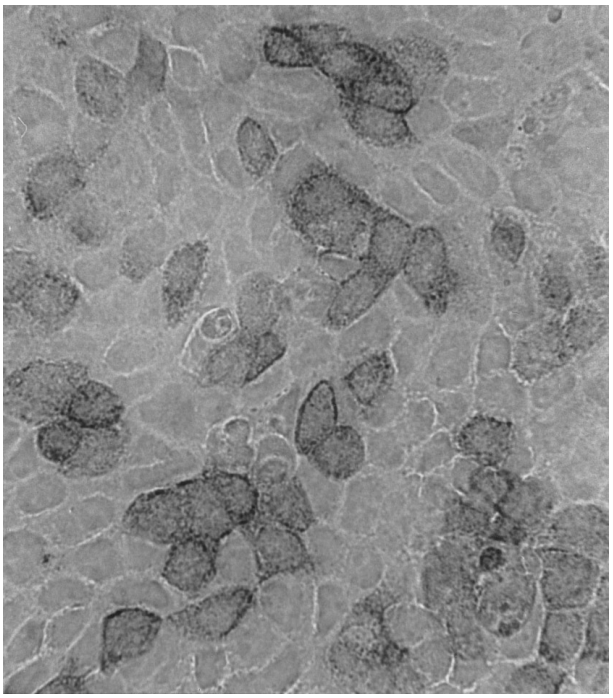


FIG. 4. (left) PCR-product amplified by RT-PCR from mRNA of Caco-2 cells using intron-spanning primers predicted to yield a 426 bp product (lane 2) Lane 1: DNA ladder size marker

FIG. 5. (right) Immunohistochemical staining of CaR in Caco-2 cells using a monoclonal anti human CaR antibody. Nonspecific staining, using specific antibody preabsorbed with the peptide against which it was raised, was minimal (not shown).

filters until 2 days after confluency in 1.80 mM $[Ca^{++}]_o$ containing DMEM, which was then replaced in the apical or/and basolateral compartment by Ca^{++}-free medium, and c-*myc* mRNA levels were determined at hourly intervals. Cells exposed to zero $[Ca^{++}]_o$ at their apical aspect for only 4 h showed an approximately threefold increase of c-myc mRNA levels compared to cells with apical $[Ca^{++}]_o$ kept constant at 1.80 mM (Fig. 3). Notably, selective withdrawal of Ca^{++} from the basolateral compartment was ineffective in up-regulating c-*myc* mRNA levels. These data strongly suggest that the sensitivity to variations in $[Ca^{++}]_o$ resides preferentially at the apical membrane of Caco-2 cells.

A number of different cell types, e. g. parathyroid, kidney and thyroidal C cells, sense variations in $[Ca^{++}]_o$ through a cell surface receptor, which has recently been cloned and characterized as a member of the family of G protein-coupled membrane receptors activating the PLC/PKC pathway (8). We therefore addressed the question whether a similar extracellular calcium-sensing receptor (CaR) might exist on the plasma membrane of Caco-2 cells.

In fact, a CaR mRNA fragment could be identified in Caco-2 cells by RT-PCR. Primers were selected from the sequence of the human CaR cDNA that were expected to amplify a 426 bp fragment coding for the last portion of the amino terminal extracellular domain of the CaR protein (amino acids 461 to 616). After fractionation of the RT-PCR products by agarose gel electrophoresis and visualization by staining with ethidium bromide, a 426 bp fragment was present (Fig. 4), which stronly suggests the presence of CaR mRNA in Caco-2 cells. Nucleotide sequencing of this product following subcloning showed 98% identity in the nucleotide sequences of the human intestinal and the human parathyroid CaR cDNA (not shown) (11).

The presence of the CaR protein on Caco-2 cells was demonstrated by immunocytochemistry with a monoclonal antibody directed against the extracellular domain of the CaR. Fig. 5 shows positive staining for the CaR protein, albeit in a mosaic pattern, with some but not all cells exhibiting staining. Perhaps CaR expression varies as a function of the cell cycle or due to paracrine interactions between Caco-2 cells in culture. The specific staining at the cell border strongly suggests that the CaR is present on the plasma membrane.

The observation that a substantial fraction of Caco-2 cells, which are able to differentiate in confluent cultures (12), exhibit the CaR, suggests that colonic epithelial cells *in vivo* may also react to changes in $[Ca^{++}]_o$, which are likely to occur mainly at the luminal aspect of the mucosal epithelium due to tight regulation of plasma $[Ca^{++}]_o$. In this regard, Whitfield (13) has suggested that in the lumen of the colonic crypts there exists a $[Ca^{++}]_o$ gradient with decreasing levels in the surface-to-crypt direction. Moreover, due to bind-

ing of Ca^{++} by mucus components on the luminal aspect of the cells, colonocytes in the proliferative compartment at the lower one-third of the crypt are likely to be exposed to extremely low levels of "free" $[Ca^{++}]_o$. This can be particularly true in situations of low oral calcium intake, which inevitably produce increased fractional absorption of Ca^{++} in the small intestine and thus a further decline in luminal $[Ca^{++}]_o$ in the large intestine. Our data strongly suggest that this can be sensed by the CaR and transduced into up-regulation of the early immediate proto-oncogene c-*myc*, although the use of a specific CaR antagonist (which is not yet available) would be necessary to prove this point unequivocally. The fact that this occurs rapidly, within 4 h of exposure to zero $[Ca^{++}]_o$ (cf. Fig. 3), implies that colonocytes can be primed for increased proliferation many times even during a relatively short period of low dietary Ca^{++} intake. We would therefore like to consider the present study as an attempt to identify molecular and cellular mechanisms which may be responsible for the facilitating effect of inadequate Ca^{++} nutrition in human colorectal carcinogenesis.

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