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# Calcium—Cell Cycle Regulator, Differentiator, Killer, Chemopreventor, and Maybe, Tumor Promoter

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**Abstract** $Ca^{2+}$ and $Ca^{2+}$-binding proteins are involved in running the cell cycle. $Ca^{2+}$ spikes and signals from integrin-activated focal adhesion complexes and $Ca^{2+}$ receptors on the cell surface along with cyclic AMP begin the cycle of cyclin-dependent protein kinases (PKs). These transiently expressed PKs stimulate the coordinate expression of DNA-replicating enzymes, activate replication enzymes, inactivate replication suppressors (*e.g.*, retinoblastoma susceptibility protein), activate the replicator complexes at the end of the $G_1$ build-up, and when replication is complete they and a $Ca^{2+}$ spike trigger mitotic prophase. Another $Ca^{2+}$ surge at the end of metaphase triggers the destruction of the prophase-stimulating PKs and starts anaphase. $Ca^{2+}$ finally stimulates cytoplasmic division (cytokinesis).

However, $Ca^{2+}$ does more than this in epithelial cells, such as those lining the colon, and skin keratinocytes. These cells also need $Ca^{2+}$, integrin signals, and only a small amount (*e.g.*, 0.05–0.1 mM) of external $Ca^{2+}$ to start DNA replication. Signals from their surface $Ca^{2+}$ receptors trigger a combination of differentiation and apoptosis ("diffpoptosis") when external $Ca^{2+}$ concentration reaches their set-points. The skin's steep, upwardly directed, $Ca^{2+}$ gradient has a low concentration in the basal layer to allow stem and precursor keratinocytes to proliferate, and higher concentrations in the suprabasal layers to trigger the differentiation-apoptosis ("diffpoptosis") mechanism that converts granular cells into protective, hard-shelled, dead corneocytes. A similar $Ca^{2+}$ gradient may exist in the colon crypt allowing the stem cell and its amplifying transit or precursor offspring to cycle in the lower parts of the crypt, while stopping proliferation and stimulating terminal differentiation in the upper crypt and flat mucosa.

Raising the amount of $Ca^{2+}$ in fecal water above a critical level reduces proliferation and thus colorectal carcinogenesis in normal rats and some high-risk humans. But during carcinogenesis the $Ca^{2+}$ sensors malfunction or their signals become ineffective: high $Ca^{2+}$ does not stop, and may even stimulate, the proliferation of initiated mutants. Therefore, $Ca^{2+}$ may either not affect, or even promote, the growth of epithelial cells in carcinogen-initiated rat colon and human adenoma patients. Clearly, a much greater understanding of how $Ca^{2+}$ controls the proliferation and differentiation of epithelial cells and why initiated cells lose their responsiveness to $Ca^{2+}$ are needed to assess the drawbacks and advantages of using $Ca^{2+}$ as a chemopreventor. 

**Key words:** Apoptosis, carcinogenesis, cell cycle, differentiation, growth factors

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Since the emerging eukaryotes of one to two billion years ago decided to use $Ca^{2+}$ as a signaller instead of ejecting it, $Ca^{2+}$ has become a very busy player in many different cellular activities. This signalling gadfly is involved in all of the phases of the cell cycle, the differentiation pro-

grams of epithelial lining cells, programmed cell death or apoptosis, a form of cellular suicide which was invented by multicellular organisms for purposes as varied as shaping body parts and killing excess or senescent cells. But as its apoptogenic involvement should tell us, $Ca^{2+}$ is dangerous: it can kill, such as when hippocampal neurones are excessively stimulated by their glutamate receptors in an ischemic brain. Nevertheless, in the last few years, people have started to look at $Ca^{2+}$ as a promising cancer chemopreventor in organs such as the colon and rectum.

## CELL CYCLES AND CHECKPOINTS

The core cell cycle consists of a $G_1$ phase during which the cell makes the first in a series of stage-specific cyclin-dependent protein kineses (PKs) that induce the coordinate expression of enzymes, accessory factors, and other components needed to replicate the chromosomes [1–3] (Fig. 1). At the end of the $G_1$ build-up, complexes of replicators known as replitases collect on the nuclear matrix-bound chromosome replication origins waiting to be switched on by the late $G_1$-specific cyclin-dependent PKs [1–3]. The S, or chromosome replication, phase is followed by the $G_2$ phase when the cell pauses again, this time to make and switch on the mitosis-triggering cyclin-dependent PKs (Fig. 1). And finally there is mitosis, those breath-taking few minutes when the cell divides into two new cells. One or both of the newborn cells may then switch off the cell cycle genes and switch out of the cycling mode.

Cells must try not to replicate damaged chromosomes, be forced to abort replication because of a shortage of replicators, prematurely initiate mitosis, start moving replicated sister chromosomes at mitotic anaphase before they have all arrived and bipolarly oriented at the spindle mid-zone, or divide before reaching a critical size

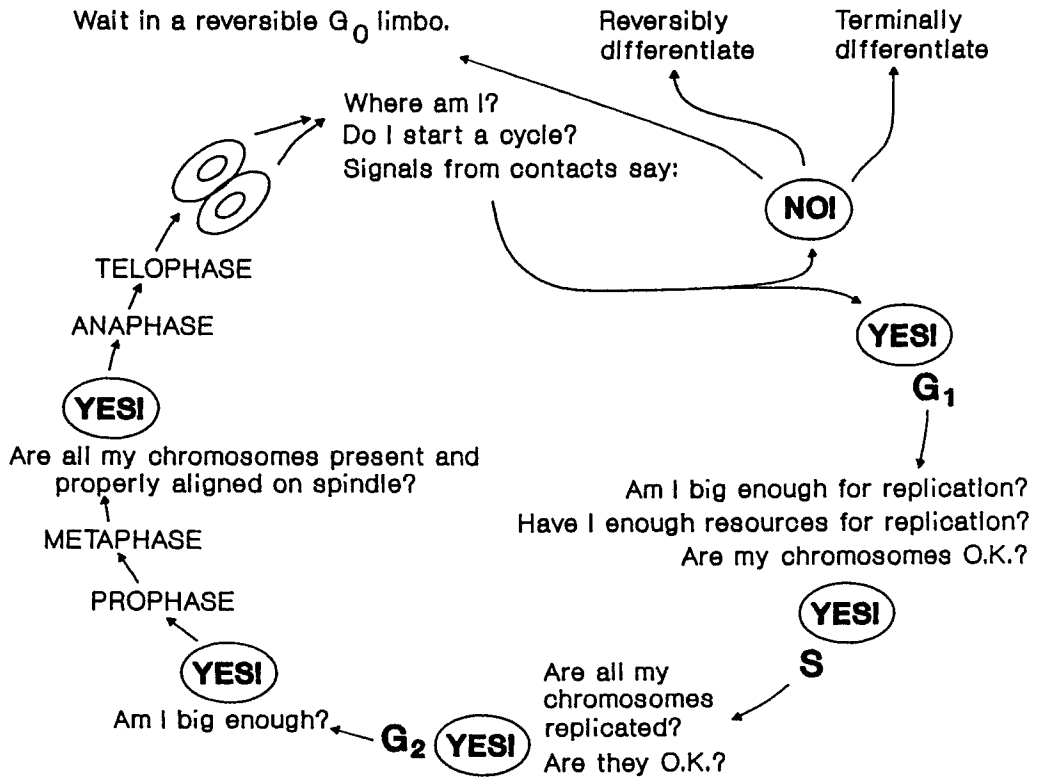



**Fig. 1.** The cell cycle, its several checkpoints, and the questions the cell must ask itself at each checkpoint. Reproduced from Whitfield [3] with permission of the publisher.

[2,3]. Therefore, a set of checkpoint sensors have evolved to delay DNA replication, prophase, or anaphase until the key requirements have been met (Fig. 1).

## $Ca^{2+}$—THE CELL CYCLE DRIVER

The most important cells for this $Ca^{2+}$ chemoprevention workshop are continuously cycling stem and precursor (or amplifying transit) cells in colon crypts and the basal layer of the epidermis. When these cells are born, they are immediately told to start the $G_1$ build-up without delay by signals from focal adhesion complexes activated by their niche-specific, substrate-binding integrin receptors and receptors for other factors such as autocrine/paracrine TGF-α [4–6]. The newborn cell starts its $G_1$ build-up with a burst of $Ca^{2+}$ oscillations [3,7] (Fig. 2), which is followed by a transient surge of cyclic AMP [3,6,8–11].

Eukaryotes from yeasts to our cells start the $G_1$ build-up with a cyclic AMP surge which subsides before the onset of DNA replication [3,6,8–11]. The cyclic AMP surge, and the cyclic AMP-dependent PKs (PKAs I and II) through which it operates, are necessary for the $G_1$ build-up and may somehow trigger the appearance of the $G_1$-specific cyclin-dependent PKs, the first of the sequentially expressed cyclin-dependent PKs that collectively make up the cell cycle engine (Fig. 3). These, in turn, start coordinate expression of replication enzymes and their accessory factors, and inactivate the Rb (retinoblastoma-susceptibility) suppressor protein that would otherwise block expression of the replication enzymes [3,11] (Fig. 3).

Normal cells need external $Ca^{2+}$ near the end of the $G_1$ build-up for the coordinate accumulation of DNA replicators. Cells such as fibroblasts, hepatocytes, and thymic lymphoblasts need around 1.0 to 1.5 mM $Ca^{2+}$, but colon cells and keratinocytes need only 0.05 to 0.1 mM (Fig. 3). If there isn't enough external $Ca^{2+}$ at this critical time, the cell will not accumulate replicators such as DNA polymerase-α, the two ribonucleo-

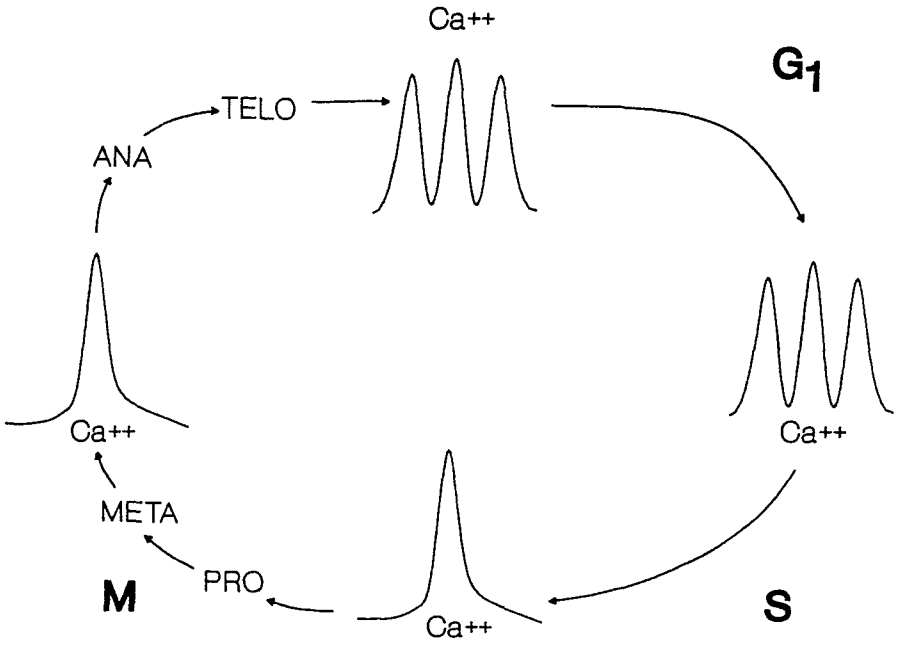



**Fig. 2.** The $Ca^{2+}$ signals that trigger various parts of the cycle. When a newborn cell in a continuously cycling population emerges from telophase, it start its internal $Ca^{2+}$ level oscillating. Near the end of the $G_1$ build-up it generates another train of $Ca^{2+}$ pulses. When the chromosomes have been replicated, the cell generates $Ca^{2+}$ spikes to help initiate mitotic prophase and anaphase. PRO, prophase; MET, metaphase; ANA, anaphase; TELO, telophase. Reproduced from Whitfield [3] with permission of the publisher.

tide reductase subunits, and thymidylate synthase [3,6,8].

By the end of the $G_1$ build-up, the cell is coordinately accumulating DNA-replicating enzymes on the nuclear matrix and matrix-associated chromosome replication origins, and planting active ribonucleotide reductase holoenzymes in the nuclear envelope to feed the replicator complexes with deoxyribonucleotide precursors when they are turned on [3,6,11].

The cell has been loading its nucleus with calmodulin which needs a $Ca^{2+}$ surge to start working. It does not have to wait long. $Ca^{2+}$ is released from stores in the nuclear envelope and soon the $Ca^{2+}$ concentration starts oscillating throughout the entire cell, and will continue oscillating for a few minutes into the S phase (Figs. 2 and 4) [7]. The cell pumps $Ca^{2+}$ into its nucleus with the $Ca^{2+}$•calmodulin-activated ATPase pumps in the nuclear envelope [3]. The nuclear $Ca^{2+}$ surge triggers the redistribution of calmodulin to the nuclear periphery. $Ca^{2+}$•calmodulin activates the nuclear envelope-associated system that transports replication-related proteins into the nucleus [3,6,11]. One of these, a 68-kDa protein, binds $Ca^{2+}$•calmodulin and assoc-

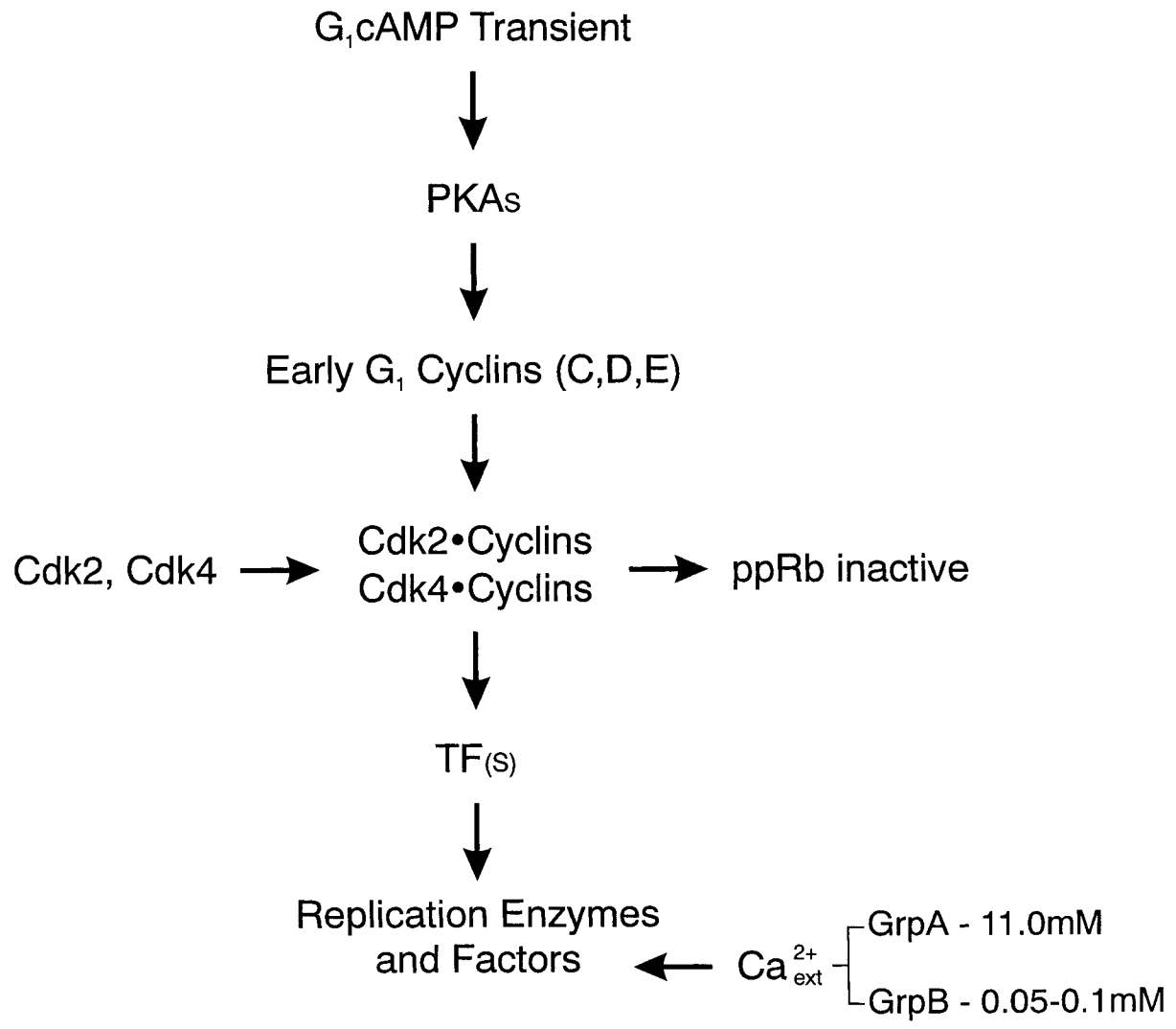



**Fig. 3.** Principal functions of cyclic AMP, the cyclic AMP-dependent protein kinases (PKAs), and $Ca^{2+}$ in the $G_1$ build-up as discussed in the text. TF(s), transcription factor(s).

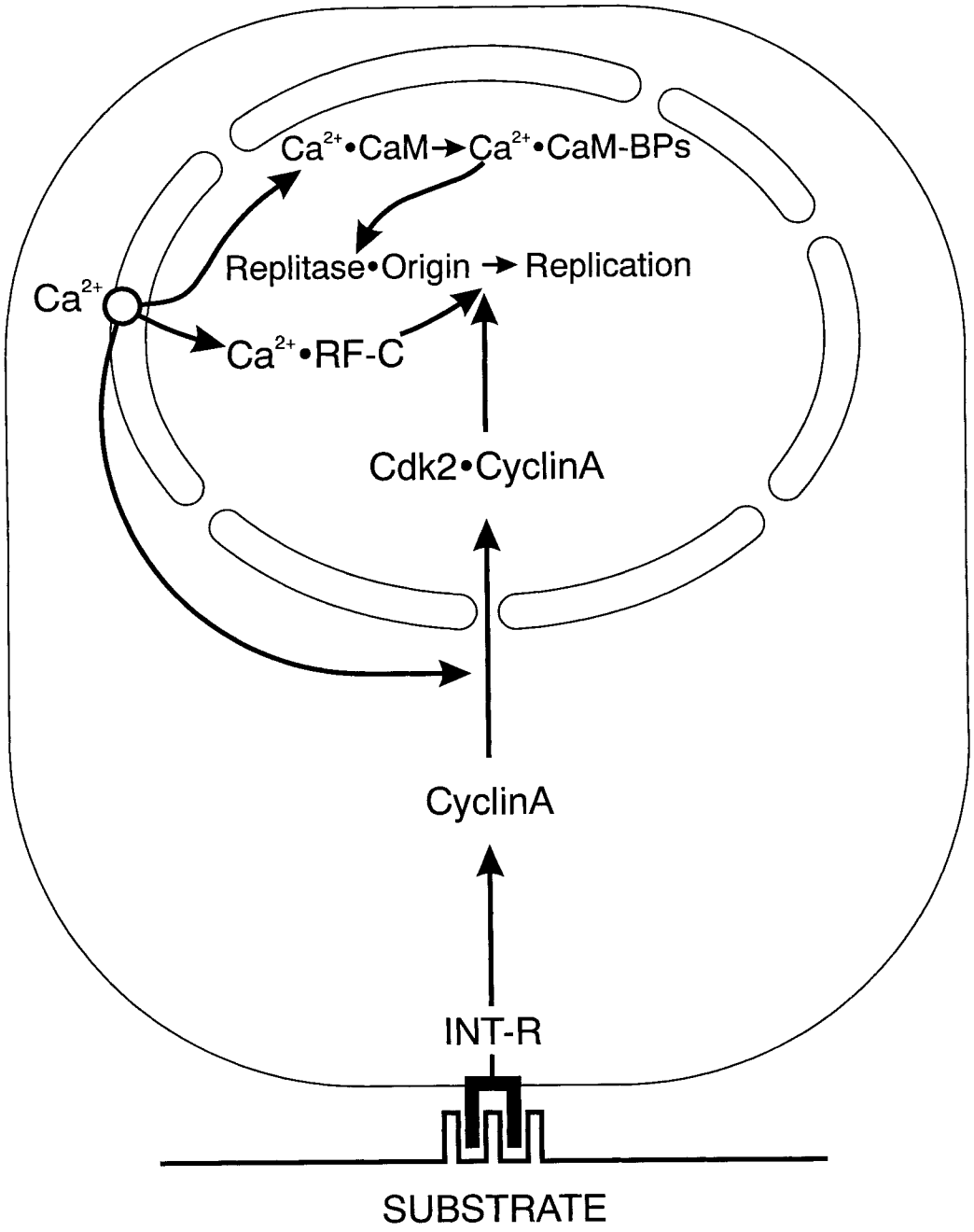



**Fig. 4.** The interactions of $Ca^{2+}$, calmodulin (cam) $Ca^{2+}$•CaM-binding proteins ($Ca^{2+}$•CaM-BPs), signals from integrin-activated focal adhesion-complexes, and a cyclin-dependent protein kinase (Cdk2•cyclin A) in the initiation of DNA replication.

iates with the replicators [3,6,11] (Fig. 4). The $Ca^{2+}$•calmodulin complexes promote the assembly and operation of the replicator complexes through the several $Ca^{2+}$•calmodulin-binding proteins associated with DNA polymerase-α (Fig. 4) [6,10,11]. The oscillating $Ca^{2+}$ also helps trigger replication by binding to annexin II (calpactin I) associated with the multisubunit RF-C (replication factor-C), that binds DNA polymerase-α to the termini of primer RNAs [3,12]. The importance of calmodulin for DNA replication is demonstrated by the rapidly reversible "freezing" of cells such as T51B rat liver cells on the threshold of the S phase by $Ca^{2+}$•calmodulin blockers [3,6,9–11].

Replication starts when a helicase on the replication origin [12] is phosphorylated by the late-$G_1$ cyclin-dependent PK, Cdk2•cyclin A (Cdk7 in the budding yeast [13]). This appeared when earlier $G_1$-specific cyclins were replaced by cyclin A, which may have been pumped into the nucleus in response to the $Ca^{2+}$ pulses (Fig. 4) [3]. The activated helicase unwinds the replication-origin DNA. The unwound origin attracts RP-A (replication protein-A), which has also been activated by being phosphorylated by Cdk2•cyclin A [2,3,14]. The DNA bound phospho-RP-A binds DNA polymerase-α primase which makes the first RNA primer [3,12].

Normal cells must be attached to a solid substrate to proliferate because the expression of the all-important cyclin A needs signals from focal adhesion complexes activated by integrin receptors such as $\alpha_5\beta_1$ [4,5] (Fig. 4). The adhesion-activated integrins activate a Src-family Y (tyrosine) PK (c-Fyn, c-Lyn, or c-Yes), which in turn activates the FAK (*f*ocal *a*dhesion *k*inase) YPK [4]. The switched-on FAK then "velcroizes" one of the focal adhesion complex's components, the 80-kDa paxilin, by phosphorylating specific Y residues [4]. Then, c-Crk, a universal adaptor protein, attaches to the "velcroized" paxilin by sticking paxillin's phospho-Y hooks into its SH2 pockets and recruits a team of signallers that triggers $Ca^{2+}$ surges, bursts of PKCs activity, and the expression of matrix-responsive genes, one of which is the gene coding for cyclin A [3–5]. Detachment from the substrate silences this signal and prevents the cell from making the Cdk2•cyclin A PKs needed to activate the replicators.

Replication will not start if the p53 protein expressed near the end of the $G_1$ build-up detects damage, making the chromosomes unfit for replication [3,7] (Fig. 1). The p53-operated mechanism will delay replication until the damage is repaired. If the damage cannot be repaired the mechanism will kill the cell by triggering apoptosis [3,15]. The value of this apoptogenic mechanism lies in the reduction of carcinogenesis by killing potentially malignant mutants.

When the chromosomes are completely replicated, the cell stops making Cdk2•cyclin A PKs and enters the $G_2$ phase. It now starts making cyclin B and Cdc2 instead of Cdk2, but keeps making cyclin A to produce a new set of cycle-engine PKs, the mitosis-triggering Cdc2•cyclin As and Cdc2•cyclin Bs [2,3,11]. There is a cyclic AMP transient surge, which, like the $G_1$ transient surge, is associated with the expression of a new set of cyclin-dependent PKs [6,8–10]. The cell also starts displaying a receptor known as HAR, the head-activator receptor, and making the autocrine/paracrine head activator peptide (first discovered in *Hydra* head) which generates a signal needed to start mitosis [3,16].

The core $G_2$ scenario goes like this [2,3]. A PK phosphorylates the emerging Cdc2s' $T^{161}$ (threonine$^{161}$) residues. This cements their union with cyclins A or B. Another PK, the dual-function "Wee-1" STYPK then phosphorylates the Cdc2s' $T^{14}$ and $Y^{15}$ residues to block their catalytic sites. This prevents the Cdc2•cyclin PKs from trying prematurely to initiate prophase while they are building up. When the inactive Cdc2•cyclin PKs reach a critical level there is another $Ca^{2+}$ spike (triggered by the HAR receptors?) (Fig. 2) which, among other things, generates the $Ca^{2+}$•calmodulin complexes that are known to be needed and may drive the inactive Cdc2•cyclin PKs into the nucleus and the waiting dual-function Cdc25-phosphatases which activate the PKs by dephosphorylating their $T^{14}$ and $Y^{15}$ residues. The activated PKs then trigger chromosome condensation and nuclear envelope breakdown of mitotic prophase.

By the end of metaphase the duplicated pairs of maximally condensed sister chromatids have lined up along the spindle equator poised to start anaphase. But the sister chromatids are still held together by residual replication tangles

[3,17]. The cell has yet another checkpoint mechanism which detects a phosphoepitope on unattached kinetochores [17] and delays anaphase until the kinetochores' phosphoepitopes have been masked or dephosphorylated when the sister chromatid pairs have all achieved a stable spindle attachment and bipolar orientation at the spindle midzone [17]. Anaphase is triggered by another $Ca^{2+}$ spike [11,17] (Fig. 2). It produces $Ca^{2+}$•calmodulin complexes that destroy the Cdc2•cyclin PKs in two ways: by stimulating the ubiquitination of the cyclins and the dephosphorylation of the Cdc2s' $T^{161}$ residues [3]. $T^{161}$ dephosphorylation causes the complexes to fall apart and ubiquitination has made the liberated cyclins attractive targets for destruction by $Ca^{2+}$•calmodulin-activated proteases, but the inactive, free Cdc2s may be spared for a while. If the chromosome-condensing Cdc2•cyclin PKs were not destroyed, the newborn cells would be unable to decondense their chromosomes and thus would be locked in mitosis. The $Ca^{2+}$ signal activates chromosome-associated topoisomerase II, which, by cutting and resolving the residual replication tangles, enables the sister chromatids to separate and start moving to their respective spindle poles [3,17]. $Ca^{2+}$ stimulates the subsequent poleward pulling of the chromosomes by their kinetochores [3]. $Ca^{2+}$ also may stimulate spindle elongation by the sliding apart of the overlapping microtubules of the two half-spindles [3].

Mitosis ends with the arrival of the chromosomes at the spindle poles, nuclear envelope assembly, chromosome decondensation, and the $Ca^{2+}$-triggered tightening of a cortical belt of actomyosin filaments that pinches the elongated parent cell into two newborn nucleus-rebuilding daughters. Depending on where they are, the available growth factors and cytokines, and signals from their neighbors, the newborn cells may start their own cycles or differentiate.

## $Ca^{2+}$—DIFFERENTIATION/APOPTOGENIC KILLER

The current best example of $Ca^{2+}$ involvement in terminal differentiation is in the skin [3,6,9–

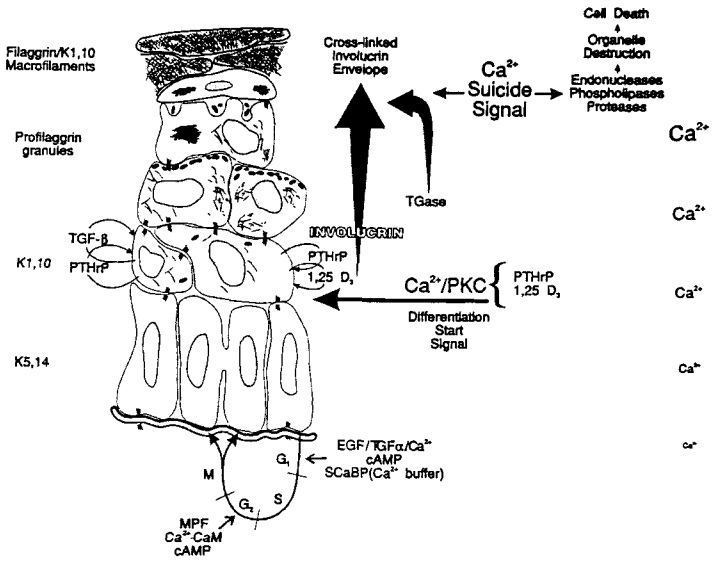



**Fig. 5.** Keratinocyte differentiation begins with continuously cycling precursor cells in the low-$Ca^{2+}$ environment at the base of the cell stack and ends at the top of the $Ca^{2+}$ gradient and near the top of the cell stack with a specialized apoptogenic ("diffpoptogenic") signal from $Ca^{2+}$ receptors that converts granular cells into dead, hard-shelled corneocytes. $Ca^{2+}$•CaM, $Ca^{2+}$•calmodulin; K 1, 5, 10, 14, keratin isotypes; PTHrP, parathyroid hormone-related protein; SCaBP, skin $Ca^{2+}$-binding protein which prevents the internal $Ca^{2+}$ concentration in basal cells from surging and prematurely triggering differentiation; TGase, transglutaminase I; $1,25D_3$, $1\alpha,25(OH)_2$-vitamin $D_3$. Reproduced from Whitfield [3] with permission from the publisher.

11,18]. The epidermis consists of epidermal proliferative units (EPUs) of upwardly rising keratinocytes with a slowly or intermittently cycling niche-bound stem cell and its more rapidly and continuously cycling precursor (or amplifying transit) cell offspring at the bottom, and the protective, dead, hard-shelled corneocytes or squames toppling off the top (Fig. 5). The epidermis maintains a steep, upwardly increasing $Ca^{2+}$ gradient (from 4 mg/kg dry weight at the bottom to 15 mg/kg at the top in human epidermis [19]) through which rising cells must pass and to which they must respond through their surface $Ca^{2+}$ receptors [3,20]. These receptors are coupled to a phospholipase-C and generate a $Ca^{2+}$ surge and burst of membrane-associated PKCs when the external $Ca^{2+}$ concentration reaches their set-point (Figs. 6 and 7).

The $Ca^{2+}$ concentration in the basal layer must be kept low (4 ± 1 mg/kg dry weight [21]), because if not the cells' $Ca^{2+}$-receptors would generate a $Ca^{2+}$- and membrane-associated PKC signal (Fig. 7) to stop proliferation and start premature cornification. As the stem cell offspring proliferate and mature, their adhesion to the basal lamina by $\alpha_5\beta_1$ and $\alpha_6\beta_4$ integrins weakens. Eventually they stop making these integrins and are pushed off the lamina into the suprabasal spinous layer by less mature cells coming behind them. Detachment from the basal lamina silences the signals from the integrin-activated focal adhesion velcroceptor complexes [4]. This stops the cells cycling because adhesion-dependent signals are needed to produce cyclin A for the Cdk2•cyclin A PKs that help start chromosome replication at the end of the $G_1$ build-up (Fig. 4) [4,5].

Upon entering the still low-$Ca^{2+}$ (3 ± 1 mg/kg dry weight in humans [19]) spinous layer, a still-growing (but not cycling) spinous cell shuts off its cell cycle genes; stops making EGF/TGF-α receptors; stops making the anti-apoptosis Bcl-2 protein which protected the intermittently cycling basal stem cells from the potentially apoptogenic, cycle-driving c-Myc protein and reactive oxygen intermediate (ROI) by-products of signals from receptors for autocrine and paracrine growth factors such as TGF-α. The cell also stops

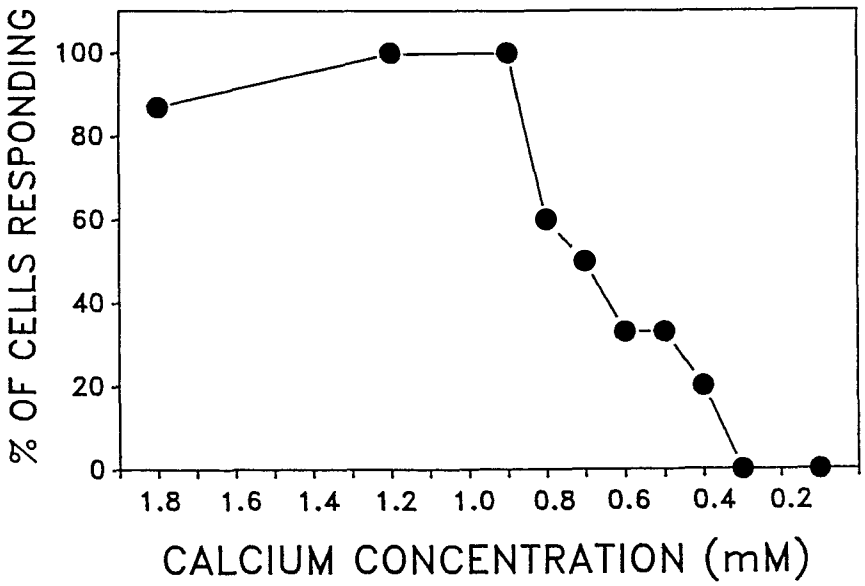



**Fig. 6.** The $Ca^{2+}$ receptors [20] on the differentiation-competent BALB/MK mouse keratinocytes trigger an internal $Ca^{2+}$ surge when the external $Ca^{2+}$ concentration rises to their set-point The mean set-point of the receptors is indicated by the external $Ca^{2+}$ concentration that induces 50% the cells to generate a $Ca^{2+}$ surge, and for these cells it appears to be 0.7 mM. The cells were cultured and the $Ca^{2+}$ response was measured as described in the legend of Figure 7.

making cycling-associated tumor-suppressing p53 protein, and lighter, proliferation-related keratins (*e.g.*, K5, K14), and starts making the heavier differentiation-related keratins (*e.g.*, K1, K10). It binds itself to its neighbors with $Ca^{2+}$-dependent cadherins and desmosomes. It starts making full-length parathyroid hormone-related protein (PTHrP) which is processed into different, potent autocrine/paracrine differentiation-promoting fragments which may stimulate the expression of other important players in differentiation including TGFs-β and $1\alpha,25(OH)_2$-vitamin $D_3$. Keratinocytes have at least two kinds of PTHrP receptors, one for the N-terminal (amino

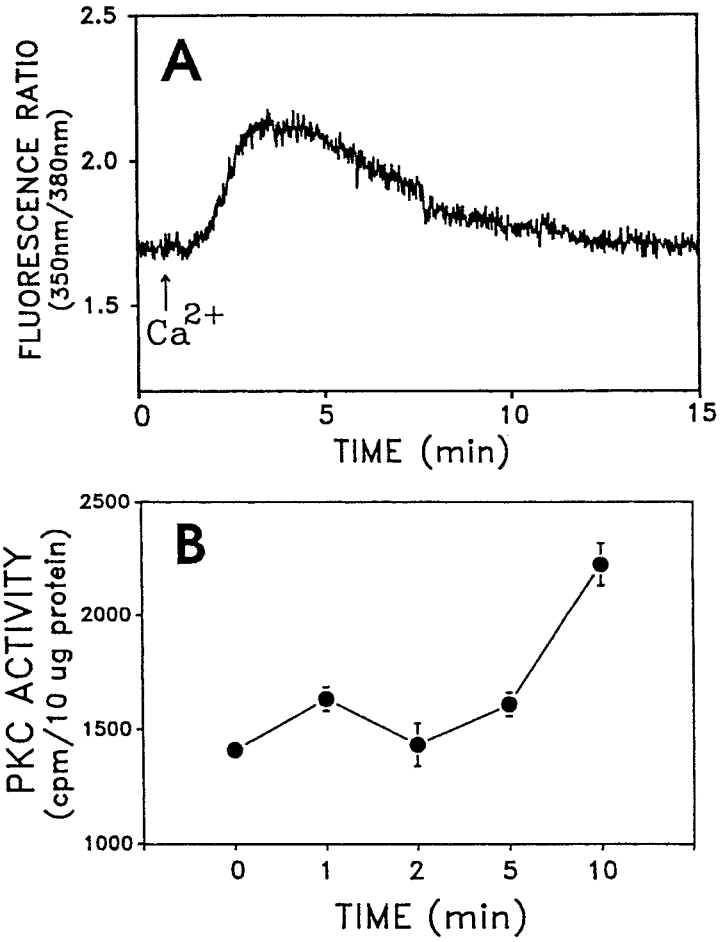



**Fig. 7.** The differentiation ("diffpoptosis")-triggering signal from $Ca^{2+}$-receptors on BALB/MK mouse keratinocytes. The cells were cultured as described by Chakravarthy *et al.* [38]. Briefly they were grown in low-$Ca^{2+}$ (0.05 mM) complete medium consisting of 10% (v/v) dialyzed fetal bovine serum (Gibco, Grand Island, NY) and 90% (v/v) Eagle's minimum essential medium (E-MEM, Biofluids, Rockville, MD) with 2 mM glutamine, 0.02 mM $Ca^{2+}$-pantothenate, 20 ng EGF/ml, and 100 units of penicillin-G/ml. The cultures were incubated at 37°C in an atmosphere of 5% $CO_2$ and 95% air. **A.** Raising the $Ca^{2+}$ concentration in the medium from 0.05 to 1.8 mM triggered a large (4.6-fold) $Ca^{2+}$ surge which peaked at 3 min. The $Ca^{2+}$ changes were measured by loading the cells with $Ca^{2+}$-sensitive fluoroprobe, fura-2. The figure is a fluorometric tracing of the ratios of the fura-2 fluorescence intensities at 505 nm after excitation at 350 and 380 nm which reflect a starting basal internal $Ca^{2+}$ concentration of 85 ± 16 nM (n=5) and a peak concentration of 394 ± 66 nM (n=5) at 3 min. **B.** The $Ca^{2+}$-triggered $Ca^{2+}$ surge was followed by a surge of membrane-associated PKCs activity. The membrane-associated PKCs activity was measured according to Chakravarthy *et al.* [38].

acids 1–34) region and one for the C-terminal (amino acids 107–111) region [3]. Very low concentrations (*e.g.*, $10^{-14}$ M–$10^{-8}$ M) of PTHrP (107–111) inhibit the proliferation of EGF-stimulated primary adult human keratinocytes [3]. By contrast, these concentrations can stimulate (2- to 3-fold) proliferation of EGF-deprived primary adult human keratinocytes [3,22].

When the cell reaches the granular layer, its external $Ca^{2+}$ concentration triples (from 3 ± 1 to 10 ± 1 mg/kg dry weight) [19]. It stops making PTHrP and down-regulates keratin expression. But now it starts the final build-up to its corneocytic demise which combines apoptosis with the expression of differentiation-specific genes ("diffpoptosis"). It starts accumulating involucrin, loricrin, the membrane-associated, $Ca^{2+}$-dependent transglutaminases 1 and 3, and profilaggrin-containing granules. When the $Ca^{2+}$ concentration around the rising, now fully loaded granular cell reaches the set point of its $Ca^{2+}$ receptors, they generate a signal that stimulates the accumulated, membrane-attached transglutaminases to weld involucrin and loricrin into a hard shell like the involucrin-containing shells of apoptotic bodies [18]; the proteolytic chopping of 350-kDa profilaggrin into 37-kDa filaggrin molecules that bundle the keratins into microfilaments; and a nuclease(s) to cut up the nuclear chromatin (Fig. 5). As soon as the $Ca^{2+}$-receptor signal has set the process in motion, the developing corneocyte uses its $Ca^{2+}$•calmodulin-activated membrane ATPases to pump the accumulated $Ca^{2+}$ into the intercellular space to maintain the gradient and prevent a loss of $Ca^{2+}$ from the body when the dead shell ultimately falls off the stack.

The colon epithelium is also continuously renewed by a slowly or intermittently cycling pluripotent stem cell with unlimited proliferative potential. In this case, a stem cell is confined to its niche at the bottom of each monoclonal crypt where it generates faster cycling, upwardly climbing precursor (or amplifying transit) cells (Fig. 8). Like the basal keratinocytes, these stem and precursor cells express the standard cell cycle genes and growth factor receptors, make the various cyclin-dependent PKs, and make anti-apoptosis Bcl-2 protein to protect themselves from the potentially apoptogenic c-Myc protein and ROI by-products of growth factor receptor signalling [3]. Unlike keratinocytes, colon cells do not stop cycling and start differentiating by lifting off the basal lamina and thereby shutting off the cycle engine. Nevertheless, at some point the precursor cells stop expressing the cell cycle genes and stop making the anti-apoptosis Bcl-2 protein and the cycle engine PKs. Instead they start differentiating into enteroendocrine and goblet cells, which tend to stay in the crypts, and absorptive cells, which move to the mouth of the crypt and onto the flat mucosa (Fig. 8). The senescent absorptive cell, like the mature granular keratinocyte, triggers its own specialized apoptotic demise (fed by the high $Ca^{2+}$ concentration in the fecal water) and is pushed, sometimes while still alive in its transglutaminase-produced shell, into the passing fecal stream by younger mature cells streaming out of the home and neighboring crypts [3,11] (Fig. 8).

Colon cells, like basal keratinocytes, need only a small amount (*e.g.*, 0.1 mM) of external $Ca^{2+}$ to proliferate optimally [3]. Also like keratinocytes, they have surface receptors [20,23,24], signals ($Ca^{2+}$ oscillations and probably a burst of PKCs activity) from which stop proliferation and trigger differentiation when the external $Ca^{2+}$ concentration reaches the 0.8 to 2.2 mM that can be found in the fecal water (Fig. 8). Therefore, the $Ca^{2+}$ concentration in the crypt's proliferation zone must somehow be kept below the receptors' set-points as it is in the basal layer of the epidermis (Fig. 8). Such a gradient might be established by $Ca^{2+}$-buffering mucins secreted by the goblet cells that tend to collect in the lower part of the crypt [9]. Thus, cycle-stopping and differentiation-starting may be the work of the signals from the $Ca^{2+}$-receptors when the precursor cells reach the point in the $Ca^{2+}$ gradient where the $Ca^{2+}$ concentration corresponds to the receptors' set-point (Fig. 8).

## $Ca^{2+}$—CHEMOPREVENTOR AND PROMOTER

Carcinogenesis involves the induction, selection, and expansion of mutant clones which are increasingly less responsive to differentiation-triggering signals. This selection process is promoted by hyperproliferation of the carcinogen-initiated cell population. The hyperproliferative activity in the colons of high-risk subjects can be

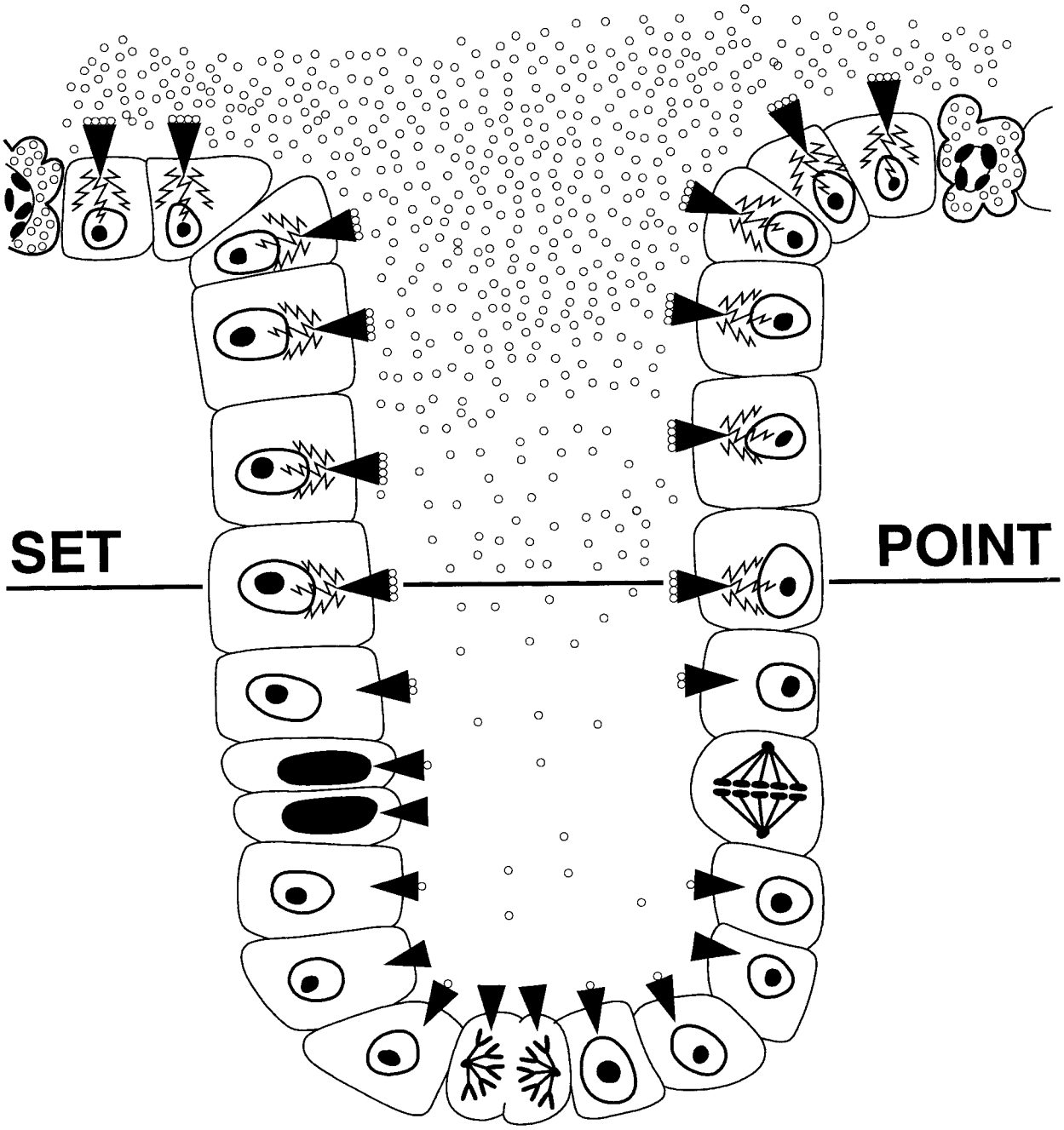



**Fig. 8.** A possible model for the control of colon cell proliferation and differentiation by $Ca^{2+}$. According to this model there is a $Ca^{2+}$ ion (o) gradient, like the epidermal $Ca^{2+}$ gradient, with the highest concentration at the mouth of the crypt and in the fecal water and the lowest concentration at the bottom of the crypt. Each colon cell is armed with $Ca^{2+}$ receptors (▲) which fire and trigger the ultimately apoptogenic differentiation program when the upwardly moving cell reaches the point in the gradient where the $Ca^{2+}$ concentration corresponds to the $Ca^{2+}$ receptors' set-points. Two $Ca^{2+}$-loaded, apoptotic cells are shown on the flat mucosa to the right and left of the mouth of the crypt.

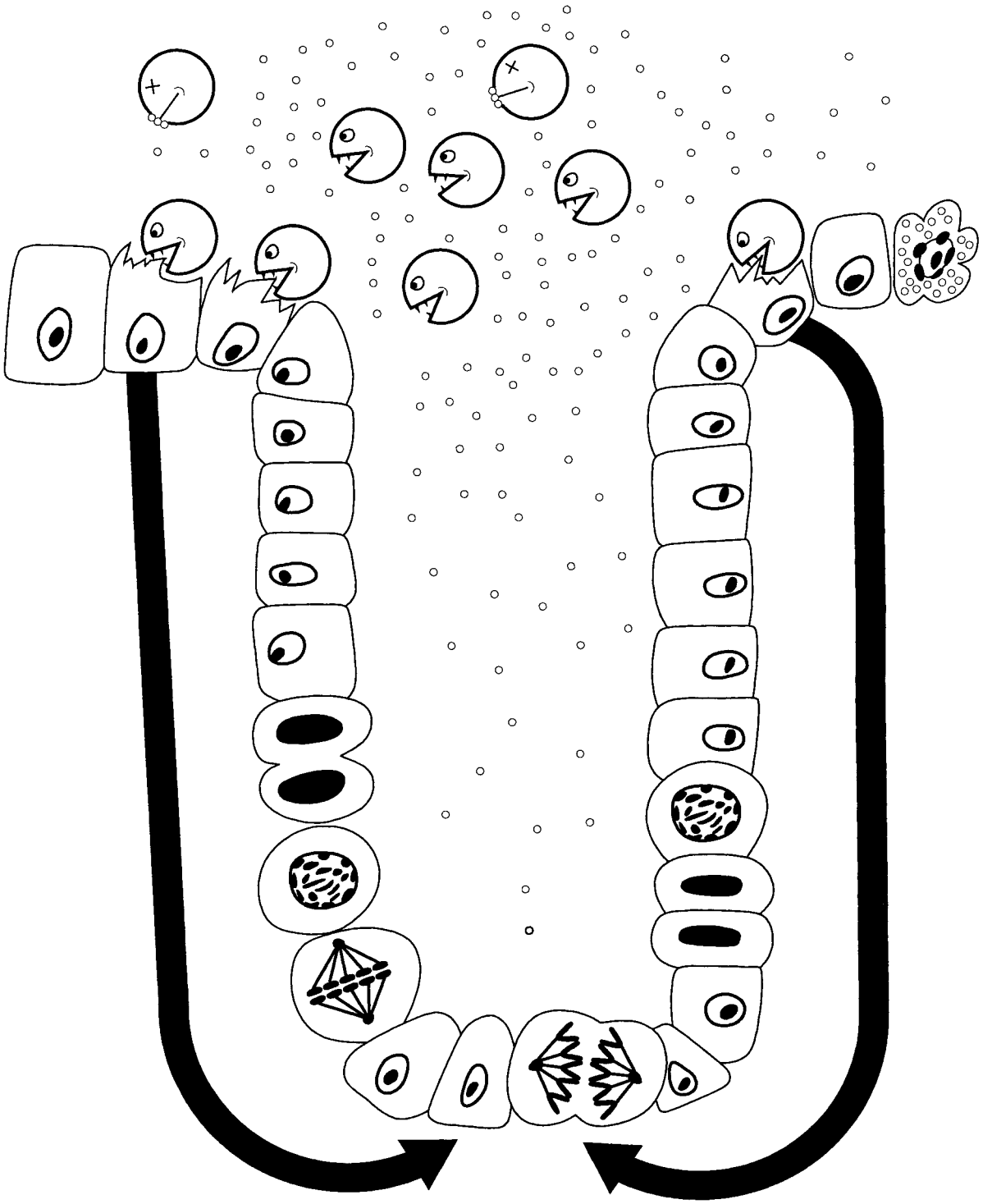


**Fig. 9.** A mechanism by which surfactant dihydroxy bile acids and free fatty acids might stimulate the proliferation of colon cells. The "Pac-Man"-like surfactants destroy cells on the mucosal surface. This sends a signal to normal or initiated stem and precursor cells at the bottom of the crypt to cycle faster. This hyperproliferation and the direct co-mutagenic and promoting actions of the surfactants would enhance the generation of malignant mutants from carcinogen-initiated adenoma cells. Note $Ca^{2+}$ is symbolized as (o).

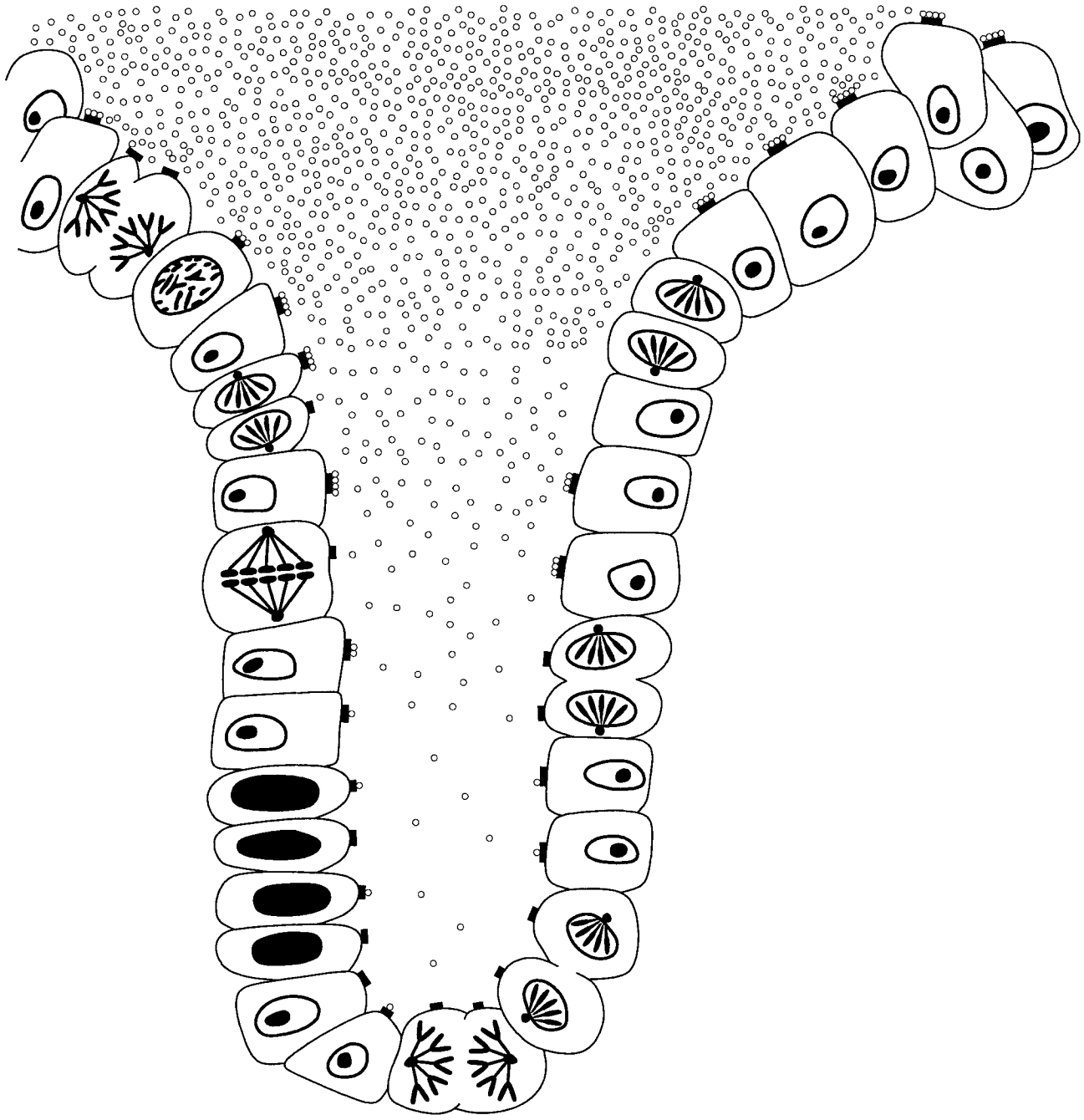
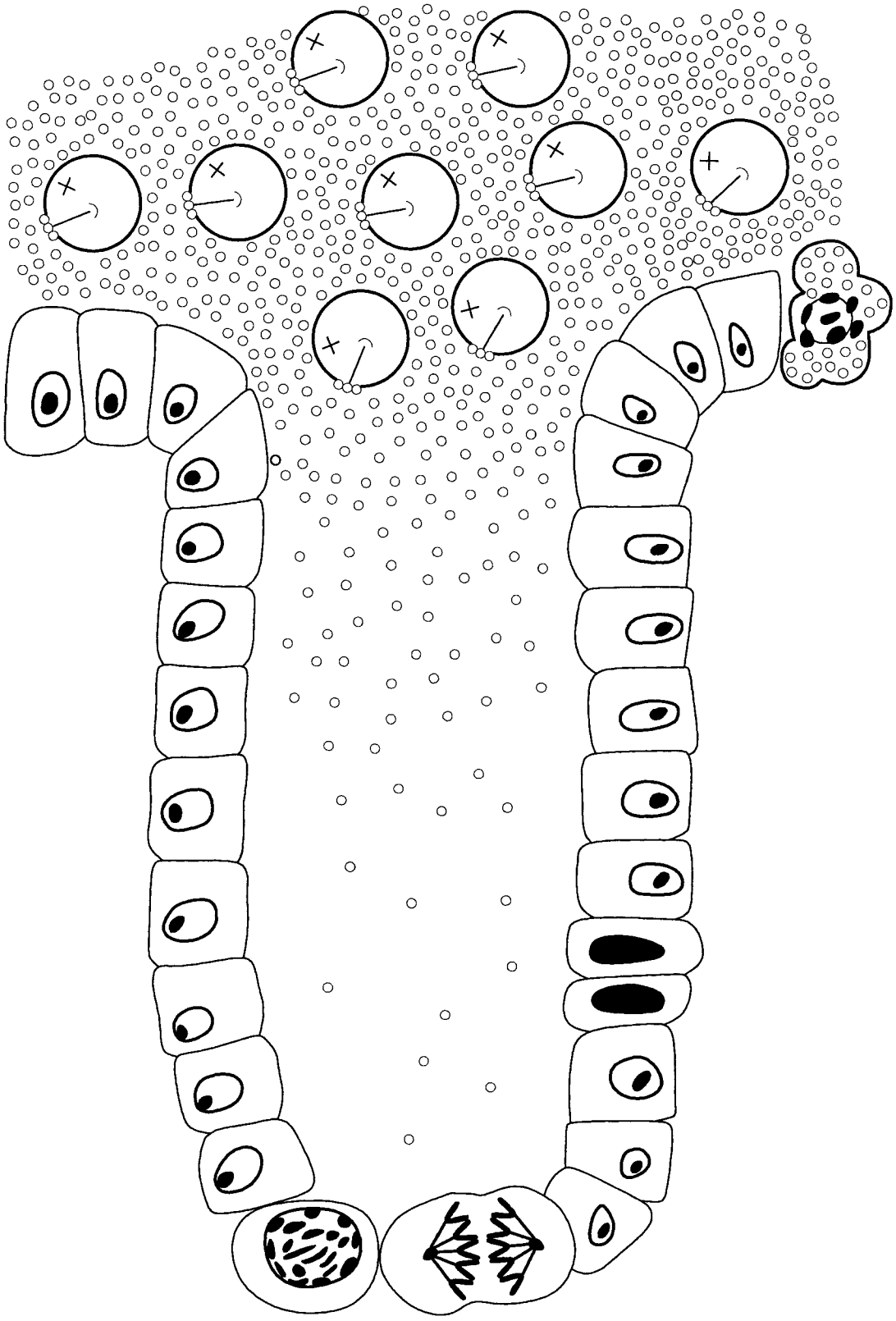


**Fig. 10.** A model for the apparently early loss of responsiveness of carcinogen-initiated colon cells to the normally cycle-stopping, differentiation-inducing $Ca^{2+}$ signals. According to this model, the emerging adenoma-producing mutants no longer express functional $Ca^{2+}$ receptors. This enables the mutant cells to cycle at all levels including the flat mucosa to produce polyps, villous adenomas, and carcinomas. Note $Ca^{2+}$ is symbolized by (o).

**Fig. 11.** One of the ways by which loading the diet, and hence the fecal water, with $Ca^{2+}$ might reduce colon cell proliferation and thus the appearance of adenomas and carcinomas. According to this model, the increased amount of $Ca^{2+}$ (o) binds to, and inactivates the co-mutagenic, tumor-promoting surfactant bile acids and long-chain fatty acids. It also reduces the cytolytic activity of the fecal water and thus the tissue-homeostatic mitogenic signalling from the mucosa to the proliferentively competent cells in the depths of the crypts.



induced by a gene mutation (*e.g.*, the deletion or loss-of-function mutations of the APC [adenomatous polyposis coli] gene on human chromosome 5q21) and/or a high level of cytolytic surfactants such as dihydroxy bile acids (chenodeoxycholic acid, deoxycholic acid) and long-chain fatty acids in fecal water [3,6,9–11,25]. The resulting cell destruction by the surfactants sends a signal down into the crypts to stimulate the proliferation of normal and initiated stem and precursor cells (Fig. 9). Combined with this indirect action is the direct co-mutagenic and tumor-promoting actions of these surfactant acids. These actions result in the upward extension of the cryptal proliferative zone, enhancement of adenoma growth and dysplasia, and the emergence of carcinomas [26] (Fig. 9). The breakdown of the terminal differentiation program in transforming clones is due to constitutive expression of cell cycle genes; overproduction of autocrine/paracrine growth factors and/or their receptors; production of an activated mutant Ras protein; persistent production of the anti-apoptosis Bcl-2 protein; inability to make the differentiation-promoting, $Ca^{2+}$-dependent cadherin cell-cell adhesion molecules; reduced expression of the differentiation-driving PKA II with continuing or enhanced expression of the proliferation-related PKA I; and loss of the p53 protein's mutation-suppressing function, facilitating the survival of malignant mutants [3].

An early, probably essential, step in colon carcinogenesis may be the loss, disabling, or uncoupling of the $Ca^{2+}$ receptors. Thus, although premalignant cells from familial adenomatous polyps and sporadic adenomas still need the small amount (0.1 mM) of external $Ca^{2+}$ that normal cells need to cycle optimally, they tend not to stop cycling and differentiate even when the external $Ca^{2+}$ concentration is raised to 2.2 mM [3,6,9–11,27]. Another common (in about 50% of cases) occurrence during carcinogenesis is the appearance of a mutant, activated Ras protein [3,6,11,14]. Such a mutant Ras, unlike its normal counterpart, potently promotes the expansion of transforming clones by further eliminating $Ca^{2+}$ controls, stimulating proliferation, and preventing the tissue-homeostatic apoptosis that can be triggered by the product of the tumor cell's overexpressed c-*myc* gene [3]. Colon carcinoma cells that ultimately emerge from the clonal selection process are completely divorced from $Ca^{2+}$: they don't need it to cycle and they don't stop cycling and differentiate when exposed to large amounts of it. Thus, adenoma and carcinoma cells can and do proliferate even in direct contact with the relatively $Ca^{2+}$-rich fecal stream on the flat mucosa which would suppress the proliferation of normal cells (Fig. 10).

It follows that reducing the hyperproliferation of colon cells caused by the high level of surfactants in the fecal water of persons consuming a typical high-fat, low-fiber, Western diet should reduce the risk of colorectal cancer. Indeed, there is consistent evidence from experiments on rats and somewhat less consistent evidence from humans, that loading the diet with $Ca^{2+}$ (*e.g.*, 2 g/day as calcium carbonate or phosphate) produces inactive complexes with the surfactant secondary bile acids and long-chain fatty acids, reduces the cytolytic activity of fecal water, prevents the appearance of mutant Ras proteins, reduces the size and number of carcinogen-induced tumors in rats, and reduces the hyperproliferative activity in the colons of high-risk humans [9,10,21, 28–33] (Fig. 11).

The key question remains as to how accessible the proliferative zones in the depths of the crypts are to $Ca^{2+}$ in the fecal water. Loading the crypts with $Ca^{2+}$ would directly reduce the proliferation and promote the differentiation of normal cells and still phenotypically normal initiated cells. But initiated, hyperplastic epithelia soon generate mutants which are unresponsive to, or stimulated by, $Ca^{2+}$. Then $Ca^{2+}$-loading would give the premalignant cells and their malignant mutants an advantage by selectively suppressing the proliferation of normal cells. Thus, $Ca^{2+}$ could be a tumor promoter like PKCs-activating TPA, which stimulates the differentiation of normal colon cells, but either does not affect or stimulates the proliferation of initiated colon cells [9,32]. This is suggested by an experiment in which 100 male Sprague-Dawley rats (maintained on a well-balanced high-fat diet) were given azoxymethane (15 mg/kg body weight/week for 2 weeks) to initiate carcinogenesis. At 12 weeks, when carcinogenesis was in full swing as indicated by extensive preneoplastic aberrant crypt formation, the rats were divided into 4 groups of 25 whose diets were adjusted (with calcium carbonate) to contain 0.1, 0.5, 1.0 or 2.0% calcium. Twelve

weeks later (24 weeks after initiating carcinogenesis) the total number of colorectal tumors was *the same* (52 to 55) in all four groups. Increasing the dietary $Ca^{2+}$ content did decrease (by 33% with 2.0% calcium) the number of tumors in the first 4 cm from the rectal end, but it dramatically increased (4-fold with 2.0% calcium) the number of tumors located 12–16 cm from the rectal end. Increasing the dietary calcium decreased the size (by about 80% with 2.0% calcium) of tumors in the first 4 cm from the rectal end, but it did not affect the size of tumors in the rest of the colon. There also are reports that dietary $Ca^{2+}$ supplementation actually stimulates proliferation of epithelial cells in the colons of patients with adenomatous polyps [12,34].

The key events in keratinocyte carcinogenesis are: become able to make cyclin A and initiate DNA replication without adhering to the basal lamina and thus become able to cycle in the suprabasal layers; constitutively express EGF/TGF-α receptors and autocrine TGF-α; lose responsiveness to anti-proliferative TGFs-β; lose the need for a small amount of external $Ca^{2+}$ to cycle; and lose responsiveness to high (*e.g.*, 1.8 mM) external $Ca^{2+}$ concentrations that trigger the specialized differentiation program ("diffpoptosis") [3,6,9–11,18]. The divorce from external $Ca^{2+}$ begins in the early stages of carcinogenesis; initiated but phenotypically semi-normal mutants with basal cell markers may respond to high external $Ca^{2+}$ concentrations by only reducing, instead of stopping, proliferation and initiating some, but not all, parts of the differentiation program [3]. Eventually, mutants appear which do not need a small amount of $Ca^{2+}$ to cycle and do not respond to the normally differentiation-triggering higher concentrations of the ion [3,6,8–11]. The response of the initiated cells to the PKCs-stimulating, tumor-promoting phorbol ester, TPA, also changes dramatically. TPA should cause cells to stop cycling and differentiate, but the initiated cells increase proliferation and don't differentiate even in the presence of a high external $Ca^{2+}$ concentration. This, of course, gives the premalignant cells a big advantage over their normal neighbors in the presence of tumor promoters. They increase their numbers while their normal neighbors stop proliferating, differentiate, and ultimately trigger the lethal, "diffpoptotic", corneocyte-forming mechanism. As the tumor promoters selectively stimulate the expansion of the neoplastic mutant clones, they stimulate a shower of ROIs via the PKCs-stimulated membrane-associated NAD(P)H oxidase that generates clastogenic agents, resulting in chromosome aberrations which can persist and contribute to the ultimate emergence of malignant mutants because of the loss, loss of function, or change of function of the tumor-suppressing p53 protein [3,8,11,15].

While loading the diet with $Ca^{2+}$ can reduce the proliferation of normal and still phenotypically normal initiated colon epithelial cells directly exposed to the high-$Ca^{2+}$ fecal water (Fig. 11), it does not affect the plasma $Ca^{2+}$ concentration, and thus does not affect keratinocyte proliferation in mouse skin [35]. The only way to get $Ca^{2+}$ to affect keratinocytes would be to apply it or a $Ca^{2+}$-ionophore (which mimics the apoptosis/differentiation-inducing $Ca^{2+}$-receptor signals [Fig. 7] by causing an intracellular $Ca^{2+}$ surge) directly to the skin. Yuspa *et al.* [36] have reported that topically applying a $Ca^{2+}$-ionophore, ionomycin, to initiated mouse skin before starting promotion, when the cells were still phenotypically normal and responsive to $Ca^{2+}$, reduced the ultimate tumor yield. After the initiated cells have become resistant to $Ca^{2+}$, high-$Ca^{2+}$ treatment should be the same as treatment with the tumor-promoting TPA whose action, like that of $Ca^{2+}$ (Fig. 7), is mediated by activated PKCs [3,6,8–11]. Indeed, Kruszewski *et al.* [37] have shown that resistance to $Ca^{2+}$ can be used to select the initiated cells from a mixed culture of normal and chemically initiated keratinocytes.

## CONCLUSION

$Ca^{2+}$ is a promising chemopreventive agent mainly for colorectal cancer. But before we can exploit its potential and assess its possible drawbacks, we must mount a major team effort to find out how it controls proliferation and, most importantly, how it triggers terminal differentiation in normal colon crypts. A clue to the diffentiation trigger is the recent discovery of surface $Ca^{2+}$ (or more correctly, polyvalent cation) receptors on colon cells [24]. Clearly, to understand how $Ca^{2+}$ stimulates terminal differentiation and why carcinogen-initiated cells become resistant to $Ca^{2+}$ requires the isolation and char-

acterization of these receptors in normal and tumor cells.

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