

# Nonsteroidal Anti-Inflammatory Drugs Attenuate Proliferation of Colonic Carcinoma Cells by Blocking Epidermal Growth Factor–Induced $\text{Ca}^{++}$ Mobilization

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Numerous studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit colorectal carcinogenesis. We have previously reported that NSAIDs, in human colonic carcinoma cells (Caco-2), attenuate epidermal growth factor (EGF)–induced cellular proliferation through a process independent of their inhibitory effects on prostaglandin synthesis. Furthermore, separate studies have also suggested that NSAIDs inhibit EGF-induced store-operated  $\text{Ca}^{++}$  influx. Thus we developed the hypothesis that NSAIDs may limit the activity of EGF by altering intracellular  $\text{Ca}^{++}$  ( $[\text{Ca}^{++}]_i$ ) mobilization. Serum-deprived Caco-2 cells were employed for all experimentation.  $[\text{Ca}^{++}]_i$  was measured with Fluo-3 and extracellular  $\text{Ca}^{++}$  influx was monitored by quenching Fluo-3 fluorescence with  $\text{Mn}^{++}$ . Proliferation was quantitated with two assays: cellular nucleic acid and total protein content. Caco-2 cells exposed to EGF demonstrated an initial increase in  $[\text{Ca}^{++}]_i$  which was blocked by neomycin, an inhibitor of  $\text{IP}_3$  generation, and the phospholipase C inhibitor U73122 but not U73343 (inactive control). This was followed by sustained extracellular  $\text{Ca}^{++}$  influx, which was attenuated with calcium-free buffer ( $-\text{Ca}^{++}$ ), the store-operated  $\text{Ca}^{++}$  channel blocker lanthanum, indomethacin, ibuprofen, and aspirin. In subsequent studies, cells were treated with either serum-free media or EGF  $\pm$  the aforementioned inhibitors, and again serum starved. Cells exposed to EGF  $\pm$  the inactive phospholipase C inhibitor U73343 demonstrated a significant increase in nucleic acid and protein. However, proliferation induced by EGF was not observed when  $[\text{Ca}^{++}]_i$  elevation was prevented by blocking either internal  $\text{Ca}^{++}$  store release via phospholipase C/ $\text{IP}_3$  or sustained  $\text{Ca}^{++}$  influx through store-operated  $\text{Ca}^{++}$  channels. Sustained  $[\text{Ca}^{++}]_i$  elevation, as induced by EGF, appears to be required for mitogenesis. These data support our premise that one mechanism whereby NSAIDs may attenuate colonic neoplasia is by blocking EGF-induced  $\text{Ca}^{++}$  mobilization. (J GASTROINTEST SURG 2000;4:150-161.)

**KEY WORDS:** Caco-2 cells, calcium, epidermal growth factor, colorectal cancer, NSAIDs

Recent laboratory, animal, epidemiologic, and clinical investigations support the concept that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both the initiation and proliferation of colorectal tumors.<sup>1-4</sup> Although several studies have suggested that endogenous prostaglandins play a large role,<sup>5-8</sup> a growing body of evidence suggests that NSAIDs may attenuate colorectal carcinogenesis independent of their inhibitory effect on prostaglandin synthesis.<sup>9-11</sup> Thus, despite significant research, the exact mechanism(s) whereby NSAIDs limit colonic neoplasia remain elusive.

We have recently investigated the effect of NSAIDs on cellular proliferation in human colonic carcinoma cells (Caco-2). Although NSAID treatment (indomethacin, ibuprofen, or aspirin) did not inhibit growth in cells treated with only serum-free medium, NSAID treatment did significantly attenuate protein and nucleic acid synthesis induced by the mitogen epidermal growth factor (EGF) through a

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process that appeared to be independent of prostaglandin synthesis inhibition.<sup>12</sup> Furthermore, separate studies have also suggested that NSAIDs inhibit EGF-induced store-operated  $\text{Ca}^{++}$  influx (SOCl). Thus we have developed the hypothesis that NSAIDs may limit the activity of EGF by altering intracellular  $\text{Ca}^{++}$  ( $[\text{Ca}^{++}]_i$ ) mobilization. The connection, however, between EGF-induced  $\text{Ca}^{++}$  mobilization and EGF-induced cellular proliferation remains poorly defined. The major objectives of the current study were to investigate the mechanisms whereby EGF elicits changes in  $[\text{Ca}^{++}]_i$ , and to determine if these effects account for its mitogenic action.

## METHODS

### Cells

Caco-2 cells were obtained from American Type Culture Collection (Rockville, Md.) at passage 15. Cells were maintained at 37° C in an atmosphere of 5%  $\text{CO}_2$  and 100% relative humidity and were split on a weekly basis at a ratio of 1:6 on reaching confluency. Cells were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium per liter of Hank's balanced salt solution (HBSS), and then plated onto either 24- or 48-well plates (Costar, Cambridge, Mass.) for experiments or into 150  $\text{cm}^2$  flasks for propagation. Cell passage was maintained between 50 and 65 and media were changed every 2 to 3 days. Caco-2 media consisted of Eagle's minimum essential medium (MEM) with nonessential amino acids supplemented with either 20% fetal bovine serum or 1% fetal bovine serum (serum-free medium), 100  $\mu\text{g}/\text{ml}$  penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin B. Serum deprivation was initiated at 80% confluency, and all experiments were performed following 48 hours of serum starvation.

### Solutions

Prior to all experiments assessing changes in  $[\text{Ca}^{++}]_i$ , media were aspirated and replaced with HBSS plus 10 mmol/L HEPES (H 8264, Sigma, St. Louis, Mo.; consisting of the following: 137 mmol/L NaCl, 5.7 mmol/L  $\text{NaHCO}_3$ , 5.3 mmol/L KCl, 1.26 mmol/L  $\text{CaCl}_2$ , and 0.8 mmol/L  $\text{MgSO}_4$ ). Experiments involving  $\text{Ca}^{++}$ -free buffer used HBSS plus 10 mmol/L HEPES and 2 mmol/L BAPTA (HBSS ( $-\text{Ca}^{++}$ ); H 6648, Sigma; consisting of 137 mmol/L NaCl, 5.7 mmol/L  $\text{NaHCO}_3$ , and 5.3 mmol/L KCl). All test compounds were dissolved in either HBSS or HBSS ( $-\text{Ca}^{++}$ ). Epidermal growth factor (EGF), neomycin sulfate (neomycin), lanthanum chloride ( $\text{La}^{++}$ ), indomethacin, acetylsalicylic acid, and ibuprofen were obtained from Sigma. The amino-

steroids U73122 and U73343 were purchased from Calbiochem (La Jolla, Calif.). Treatment with the preceding antagonists/inhibitors involved a 20-minute preincubation time followed by the addition of the respective inhibitor to all subsequent solutions within treatment groups.

### Measurement of $[\text{Ca}^{++}]_i$ and Extracellular $\text{Ca}^{++}$ Influx

Changes in intracellular  $\text{Ca}^{++}$  concentration ( $[\text{Ca}^{++}]_i$ ) were quantitated as previously described<sup>13</sup> using the single wavelength  $\text{Ca}^{++}$  indicator Fluo-3 (Fluo-3, AM; Molecular Probes, Eugene, Ore.). Prior to loading with Fluo-3, cells were washed twice with HBSS. Fluo-3 was initially dissolved in Pluronic F-127 (20% solution in dimethylsulfoxide; Molecular Probes) to make a 1 mmol/L working solution and subsequently added to HBSS plus 1% fetal bovine serum for a final loading concentration of 4  $\mu\text{mol}/\text{L}$ . Cells were then loaded with Fluo-3 for 50 minutes at 25° C in an atmosphere of 5%  $\text{CO}_2$  and 100% relative humidity.

Caco-2 cells were then washed three times to ensure removal of all unloaded Fluo-3 and control and test solutions were added to the respective wells. At each time point, intracellular  $\text{Ca}^{++}$  concentration was calculated using the following equation:

$$[\text{Ca}^{++}]_i \text{ (nmol/L)} = K_d \frac{(F - F_{\min})}{(F_{\max} - F)}$$

where  $F_{\min} = 1.25 F_{\text{MnCl}_2} - 0.25 F_{\max}$  and  $K_d = 400 \text{ nmol/L}$ .<sup>14</sup> The maximum fluo-3 signal, or  $F_{\max}$ , was determined by permeabilizing Caco-2 cells with 50  $\mu\text{mol}/\text{L}$  digitonin (Sigma). The Fluo-3 signal was quenched to obtain  $F_{\text{MnCl}_2}$  using 2 mmol/L  $\text{MnCl}_2$  and 50  $\mu\text{mol}/\text{L}$  digitonin. Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN; 50  $\mu\text{mol}/\text{L}$ , Molecular Probes) was used in all solutions as a heavy metal scavenger.<sup>15</sup>

It is well accepted that manganese ( $\text{Mn}^{++}$ ) can be used as a  $\text{Ca}^{++}$  surrogate to estimate extracellular  $\text{Ca}^{++}$  influx through the plasma membrane.<sup>16</sup> In separate experiments,  $\text{Mn}^{++}$  uptake was monitored by quenching Fluo-3 fluorescence with the addition of 2 mmol/L  $\text{MnCl}_2$  to all solutions (control and experimental). Data are presented as mean relative fluorescence.

Continuous fluorescent signals during both protocols were quantitated using a CYTOFLUOR II fluorescent multiwell plate reader (PerSeptive Biosystems, Framingham, Mass.) employing 485 nm and 530 nm as the excitation and emission spectra, respectively.

Cells were maintained throughout the experiments at a temperature of 37° C with a heated stage.

### Measurement of Cellular Proliferation

Cellular proliferation was estimated by measuring nucleic acid and, in separate samples, protein. A CyQUANT proliferation assay kit (C 7026; Molecular Probes) was employed to measure total nucleic acid. This assay relies on a green fluorescent dye, which exhibits strong fluorescent enhancement when bound to cellular nucleic acids. Protein levels were quantitated with a NanoOrange protein quantitation kit (N 6666; Molecular Probes). This assay is based on the binding of a fluorescent dye to the detergent coating and hydrophobic regions of proteins. For both assays, a CYTOFLUOR II fluorescent multiwell plate reader (PerSeptive Biosystems) was utilized employing the following spectra: 485 nm (excitation) and 530 nm (emission) for CyQUANT or 485 nm (excitation) and 590 nm (emission) for NanoOrange. Data for both protein and nucleic acid are presented as mean relative fluorescence.

### Experimental Design

We initially verified our *in vitro* model of cellular proliferation and compared nucleic acid and protein synthesis induced by EGF to 20% fetal bovine serum. The next experiment was designed to determine the optimal time duration of EGF treatment required to elicit cellular proliferation. In the third and fourth experiments, we investigated the mechanism(s) whereby EGF elicits changes in  $[Ca^{++}]_i$ . The role of phosphoinositide-specific phospholipase C (PLC) and subsequent inositol 1,4,5-trisphosphate ( $IP_3$ ) generation was determined using the aminosteroid U73122, an inhibitor of PLC catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ), its inactive analogue U7343, and neomycin (an inhibitor of  $IP_3$  generation).<sup>17,18</sup> The mechanism of sustained influx of extracellular  $Ca^{++}$  was investigated with  $Ca^{++}$ -free buffer and the store-operated  $Ca^{++}$  channel (SOCC) blocker lanthanum ( $La^{+++}$ ).<sup>16</sup> Inhibition of EGF-induced SOCI by NSAIDs was also verified employing the current experimental conditions. In subsequent studies, cells were serum starved (48 hours), pretreated (20 minutes) with either HBSS or the aforementioned inhibitors, treated (30 minutes) with either serum-free medium (MEM) or EGF, and again serum starved (48 hours) to determine what effect EGF-induced  $Ca^{++}$  mobilization may have on EGF-induced nucleic acid and protein synthesis. Finally, to rule out the possibility that any inhibition of EGF-induced mitogenesis may be a result of inhibitor ac-

tivity unrelated to EGF-induced  $Ca^{++}$  mobilization, cells were serum starved (48 hours), treated (30 minutes) with either serum-free medium (MEM) or EGF, subsequently treated (20 minutes) with the aforementioned inhibitors, and again serum starved (48 hours).

### Statistics

Statistical evaluation was performed by analysis of variance with a Scheffe post hoc test. Data ( $n = 8$  to 12 per group) are reported as mean  $\pm$  standard error of the mean. A  $P$  value  $<0.05$  was taken to represent statistical significance.

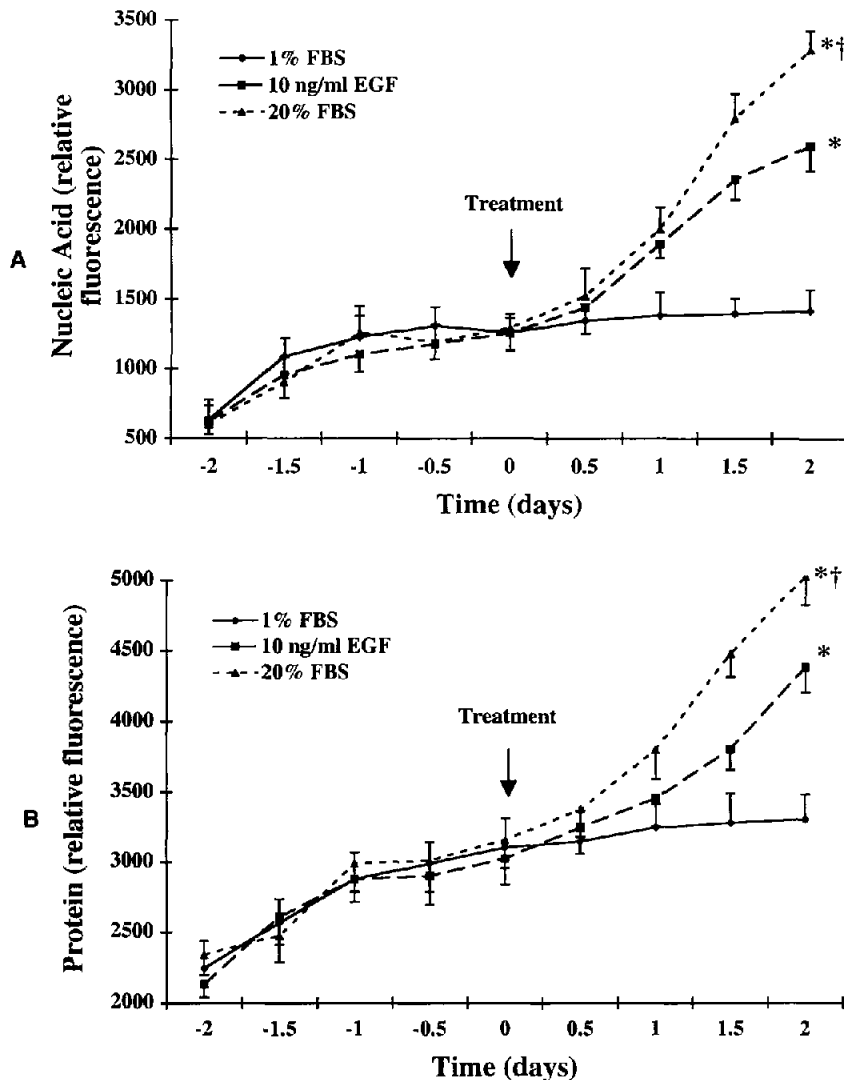
## RESULTS

### EGF-Induced Cellular Proliferation

Preliminary data suggested that concentrations of EGF ranging from 10 to 1000 ng/ml elicited elevated but similar growth rates in treated Caco-2 cells, whereas lower EGF concentrations (1 ng/ml) did not appear to induce cellular proliferation (data not shown). Thus the lowest mitogenic concentration of EGF (10 ng/ml), a concentration considered to be physiologic, was employed for all subsequent experimentation. Caco-2 cells serum deprived (1% fetal bovine serum) for 48 hours demonstrated a plateau of nucleic acid and protein levels. Subsequent treatment with EGF (10 ng/ml) initiated a significant increase in both nucleic acid and, at a later time point, protein synthesis when compared to cells subsequently treated with only 1% fetal bovine serum. However, cells subsequently treated with 20% fetal bovine serum demonstrated higher nucleic acid and protein levels when compared to cells exposed to EGF. These data are shown in Fig. 1, *A* and *B*, and suggest that although EGF is a potent mitogen for quiescent cells, greater proliferation is induced with 20% fetal bovine serum.

### Effect of Treatment Duration

In separate studies, Caco-2 cells were serum deprived for 48 hours, treated with EGF for variable time periods, and again serum deprived for a time period of 48 hours minus the EGF treatment duration. Cells treated with EGF for less than 20 minutes demonstrated nucleic acid and protein levels similar to those of control cells. Interestingly, cells treated for longer incubations (30 minutes to 48 hours) achieved increased and similar growth rates. These data are depicted in Fig. 2 and demonstrate that the signal whereby EGF elicits mitogenesis occurs within a relatively short period of time.



**Fig. 1.** Nucleic acid (A) and protein (B) levels in Caco-2 cells following 48 hours of serum deprivation and subsequent treatment with either 1% fetal bovine serum (FBS), epidermal growth factor (EGF), or 20% FBS (\* =  $P < 0.01$  vs. cells treated with MEM alone; † =  $P < 0.01$  vs. cells treated with EGF;  $n = 12$  per group).

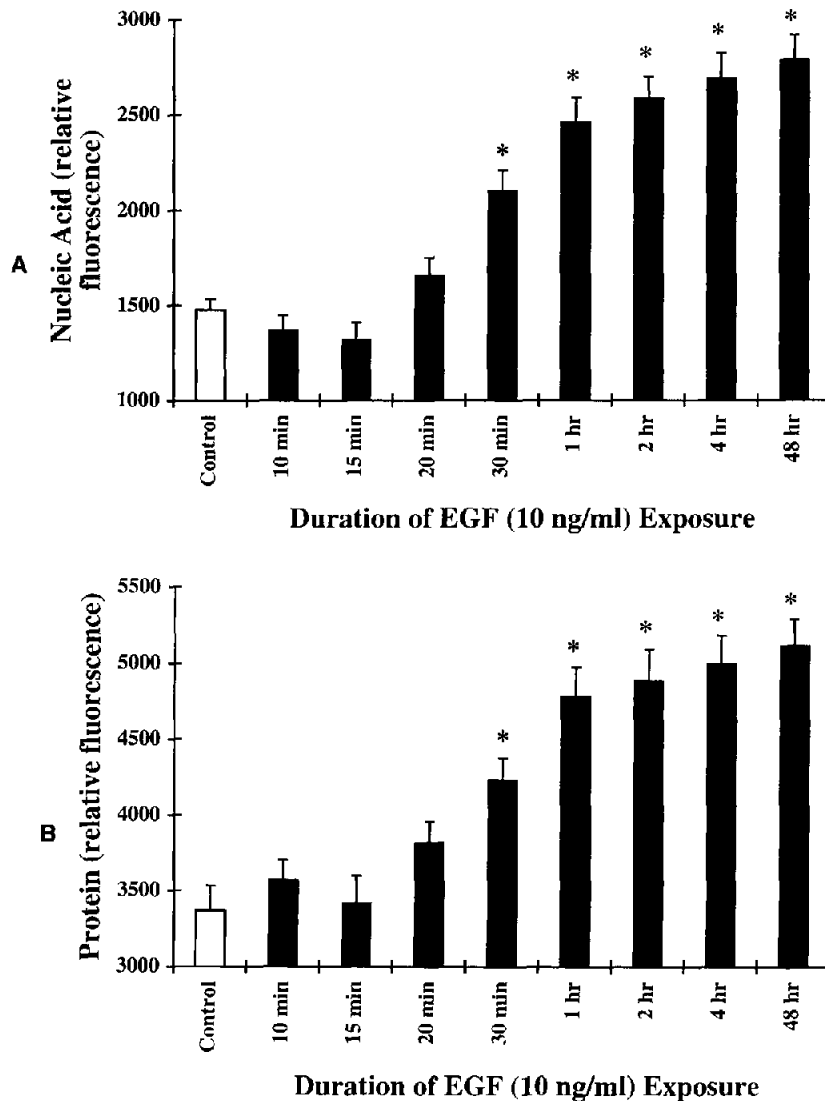
### EGF-Induced $\text{Ca}^{++}$ Mobilization (Intracellular $\text{Ca}^{++}$ Stores)

Previous data have suggested that EGF may initially increase  $[\text{Ca}^{++}]_i$  through the release of intracellular  $\text{Ca}^{++}$  stores. Caco-2 cells, following 48 hours of serum deprivation, exposed to 10 ng/ml EGF demonstrated an initial increase in  $[\text{Ca}^{++}]_i$  present within 2 to 3 minutes. Cells pretreated with neomycin (100  $\mu\text{mol/L}$ ; 20 minutes) or U73122 (1  $\mu\text{mol/L}$ ; 20 minutes), and subsequently treated with EGF, displayed no such elevation in  $[\text{Ca}^{++}]_i$ , and their levels were noted to be similar to control values. Cells pretreated with the inactive analogue U73343 (1  $\mu\text{mol/L}$ ; 20

minutes), however, and subsequently exposed to EGF, demonstrated no difference with regard to changes in  $[\text{Ca}^{++}]_i$  when compared to cells treated with only EGF. These data are shown in Fig. 3 and demonstrate that the initial increase in intracellular  $\text{Ca}^{++}$  content induced by EGF involves the release of intracellular  $\text{Ca}^{++}$  stores via a PLC- and  $\text{IP}_3$ -related mechanism.

### EGF-Induced $\text{Ca}^{++}$ Mobilization (SOCl)

Following the initial increase in  $[\text{Ca}^{++}]_i$ , Caco-2 cells exposed to EGF demonstrated a sustained elevation lasting up to 20 minutes (data not shown). Pre-



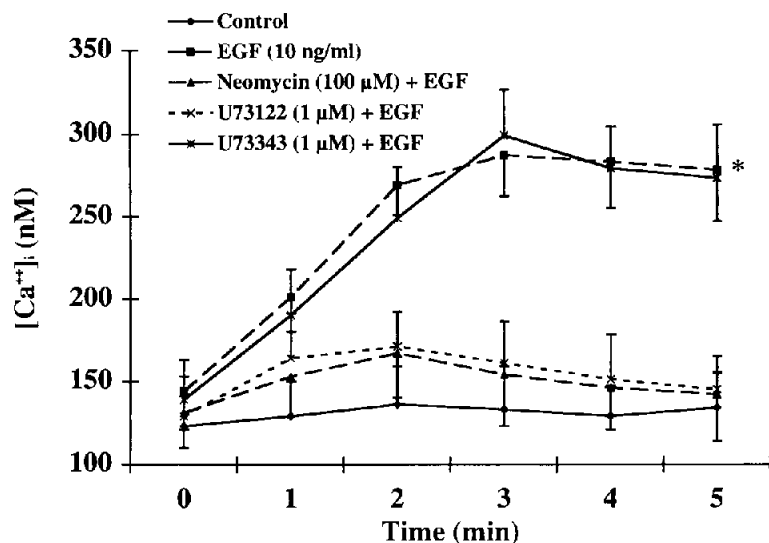
**Fig. 2.** Effect of epidermal growth factor (EGF) treatment duration on nucleic acid (A) and protein (B) levels in Caco-2 cells following 48 hours of serum deprivation (\* =  $P < 0.01$  vs. control;  $n = 6$  to 12 per group).

liminary data have suggested that this plateau is a result of the influx of extracellular  $\text{Ca}^{++}$  through SOCCs.<sup>19</sup> Sustained intracellular  $\text{Ca}^{++}$  elevations (4 to 12 minutes) were not observed in cells treated with 10 ng/ml EGF in the absence of extracellular  $\text{Ca}^{++}$ . Quenching of Fluo-3 fluorescence by  $\text{Mn}^{++}$  was first evident 8 minutes after EGF exposure, which suggests the influx of extracellular  $\text{Ca}^{++}$  over this time period. Pretreatment of Caco-2 cells with the SOCC blocker  $\text{La}^{+++}$  (25  $\mu\text{mol/L}$ ; 20 minutes), alone, did not significantly affect intracellular  $\text{Ca}^{++}$  levels (data not shown) but did inhibit both the sustained intracellular  $\text{Ca}^{++}$  plateau and  $\text{Mn}^{++}$  uptake following EGF treatment. Pretreatment of cells with nondamaging, physiologically appropriate concentrations of indomethacin (5  $\mu\text{mol/L}$ ; 20 minutes), ibuprofen (10  $\mu\text{mol/L}$ ; 20 minutes), and acetylsalicylic acid

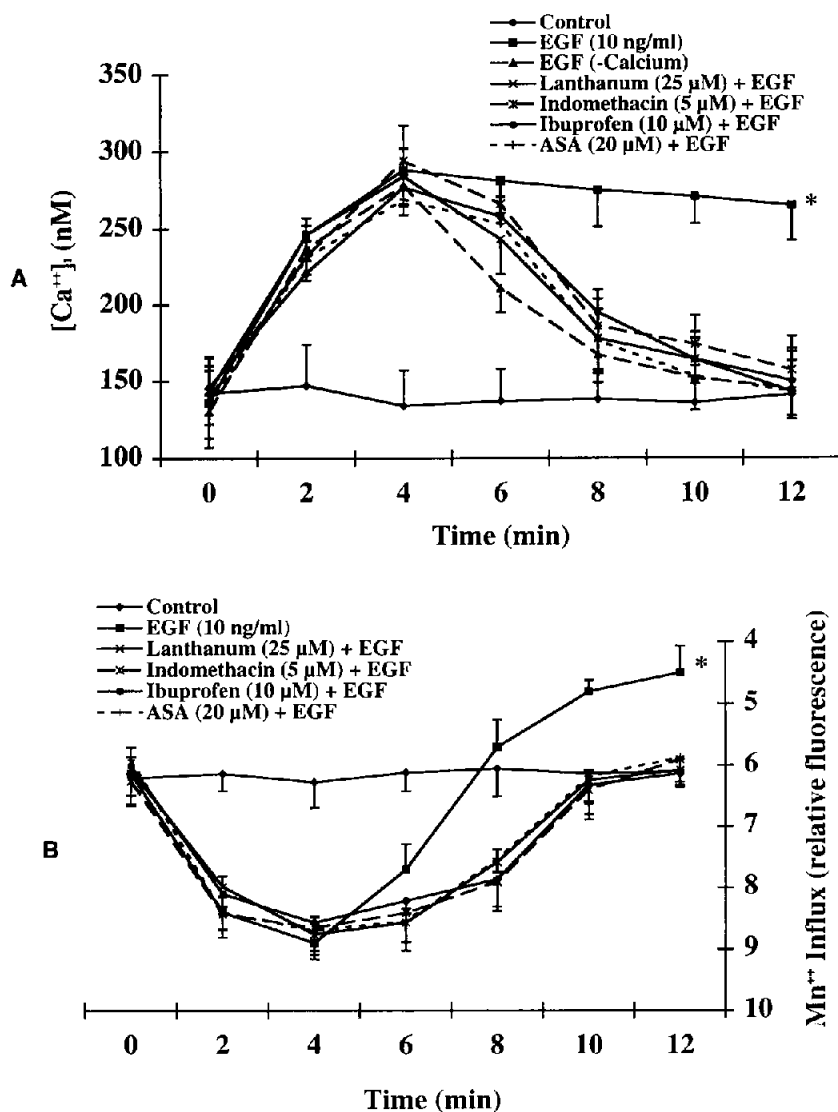
(20  $\mu\text{mol/L}$ ; 20 minutes) equally inhibited the influx of extracellular  $\text{Ca}^{++}$  as determined by both  $[\text{Ca}^{++}]_i$  measurements and  $\text{Mn}^{++}$  influx. These data, depicted in Fig. 4, are consistent with the concept that the sustained  $\text{Ca}^{++}$  elevation, as induced by EGF, is mediated by the influx of extracellular  $\text{Ca}^{++}$  through SOCCs. Furthermore, EGF-induced SOCI appears to be inhibited by NSAIDs in a manner very similar to the SOCC blocker  $\text{La}^{+++}$ .

#### Effect of $\text{Ca}^{++}$ Signaling on EGF-Induced Mitogenesis

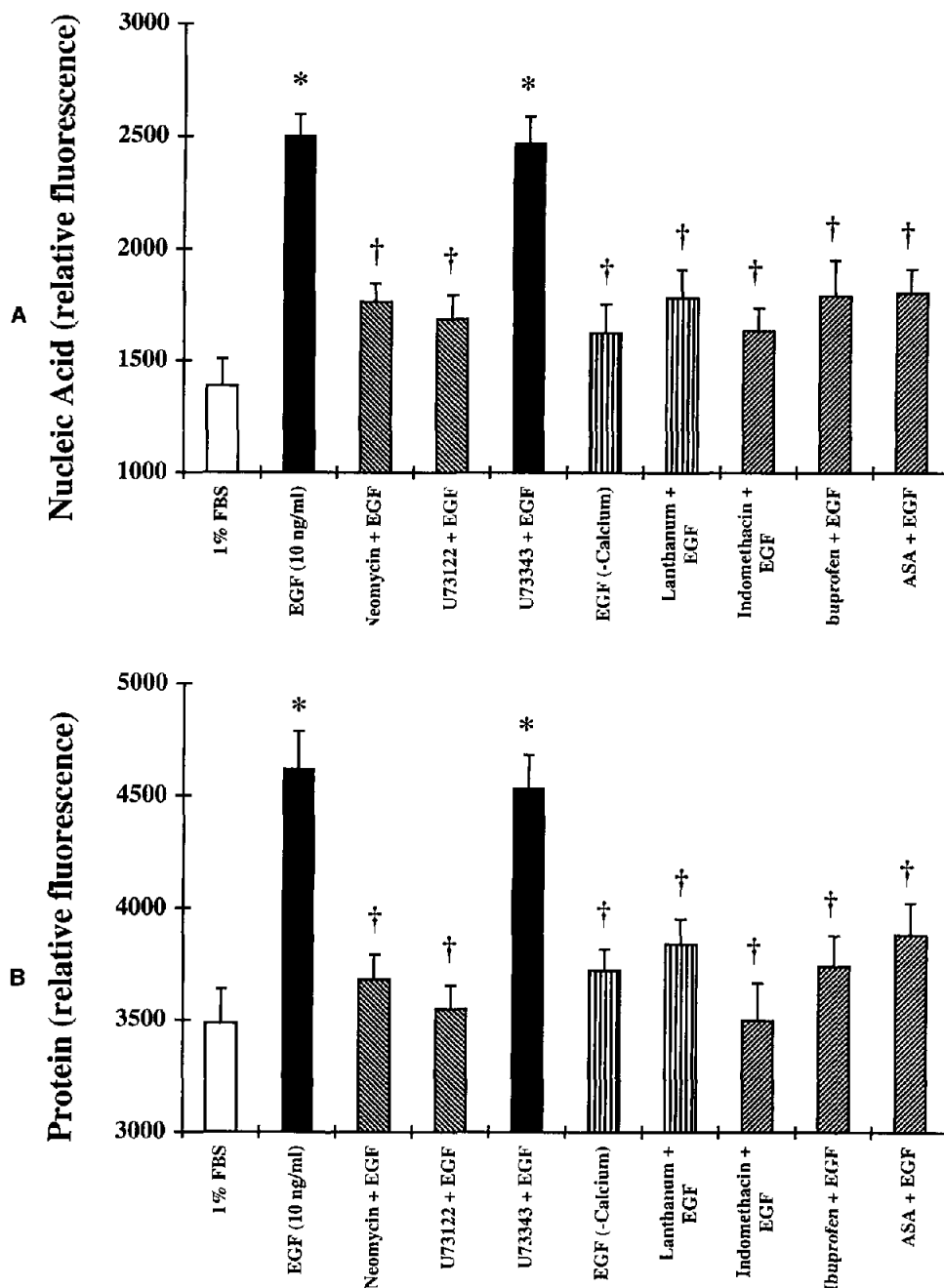
Caco-2 cells, following 48 hours of serum deprivation and subsequent pretreatment (20 minutes) with or without the aforementioned inhibitors, were then exposed to either 1% fetal bovine serum or 10 ng/ml



**Fig. 3.** Changes in intracellular calcium content in response to epidermal growth factor (*EGF*) alone or following pretreatment with neomycin (an inhibitor of  $IP_3$  generation), the PLC inhibitor U73122, or its inactive analogue U73343 (\* =  $P < 0.01$  vs. control;  $n = 6$  to 12 per group).



**Fig. 4.** Effect of pretreatment with either  $La^{3+}$  or various NSAIDs on changes in intracellular calcium concentration (A) or manganese influx (B) induced subsequently by epidermal growth factor (*EGF*) (\* =  $P < 0.01$  vs. control;  $n = 6$  to 12 per group).

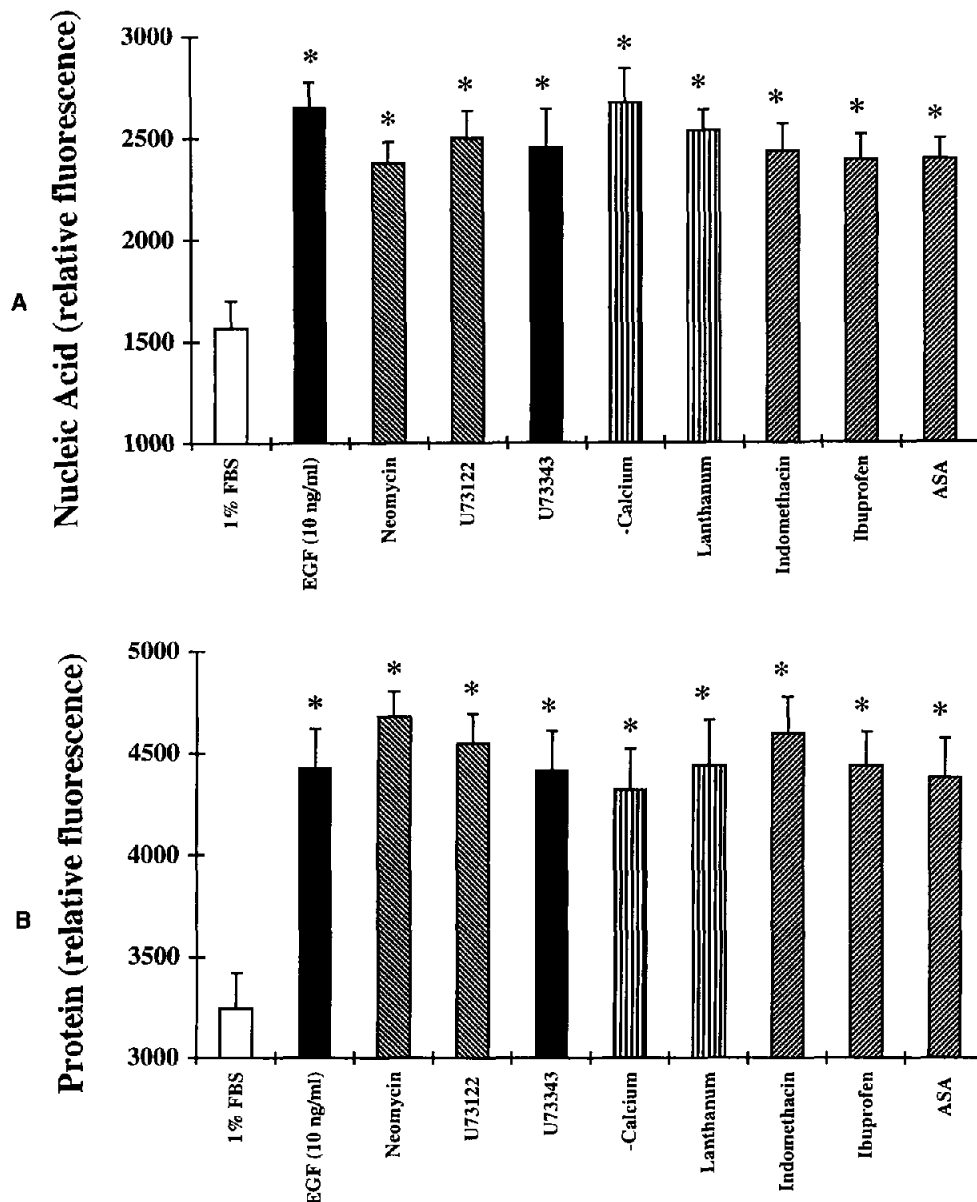


**Fig. 5.** Effect of pretreatment with either neomycin (an inhibitor of  $IP_3$  generation), the PLC inhibitor U73122 or its inactive analogue U73343,  $La^{+++}$ , or various NSAIDs on nucleic acid (**A**) and protein (**B**) synthesis induced by epidermal growth factor (EGF) following 48 hours of serum deprivation (\* =  $P < 0.01$  vs. 1% fetal bovine serum (FBS) treatment; † =  $P < 0.01$  vs. EGF treatment;  $n = 12$  per group).

EGF. Cells exposed to EGF, with or without pretreatment with the inactive PLC inhibitor U73343, demonstrated a significant increase in nucleic acid and protein levels when compared to cells subsequently treated with only 1% fetal bovine serum. In contrast, EGF-induced nucleic acid or protein synthesis was not observed when any of EGF's effects on changes in intracellular  $Ca^{++}$  were prevented: internal store

release via PLC and  $IP_3$  (neomycin or U73122 pretreatment) or sustained extracellular  $Ca^{++}$  influx through SOCCs ( $Ca^{++}$ -free buffer,  $La^{+++}$ , indomethacin, ibuprofen, or acetylsalicylic acid pretreatment). These data are depicted in Fig. 5, A and B.

Finally, Caco-2 cells were serum starved (48 hours), treated (30 minutes) with either 1% fetal bovine serum or 10 mg/ml EGF, subsequently treated (20



**Fig. 6.** Effect of posttreatment with either neomycin (an inhibitor of IP<sub>3</sub> generation), the phospholipase C inhibitor U73122 or its inactive analogue U73343,  $\text{La}^{+++}$ , or various NSAIDs on nucleic acid (**A**) and protein (**B**) synthesis induced by epidermal growth factor (EGF) following 48 hours of serum deprivation (\* =  $P < 0.01$  vs. 1% fetal bovine serum (FBS) treatment;  $n = 12$  per group).

minutes) with the aforementioned inhibitors, and again serum starved (48 hours). Cells treated with EGF, as previously shown, demonstrated significantly increased nucleic acid and protein synthesis when compared to cells treated with only MEM. However, when cells were exposed to EGF and subsequently exposed to the aforementioned inhibitors of EGF-induced  $\text{Ca}^{++}$  mobilization, we observed no difference with regard to either nucleic acid or protein levels when compared to cells treated with EGF alone. These data, depicted in Fig. 6, suggested that inhibi-

tion of EGF-induced mitogenesis, as shown in Fig. 5, was a result of inhibitor activity directly related to EGF-induced  $\text{Ca}^{++}$  mobilization.

## DISCUSSION

Previous studies from our laboratory have suggested that NSAIDs inhibit EGF-induced mitogenesis by blocking EGF-induced  $\text{Ca}^{++}$  signaling. Thus we speculated that EGF-induced  $\text{Ca}^{++}$  mobilization was a prerequisite for subsequent cellular prolifera-



tion. The current report strongly suggests that changes in  $\text{Ca}^{++}$  concentration, as induced by EGF, is required for mitogenesis. Several lines of evidence support this contention. The signal whereby EGF initiates the process of proliferation occurs within a relatively short period of time (30 minutes). This early signal appears to involve the initial release of  $\text{Ca}^{++}$  through the release of intracellular  $\text{Ca}^{++}$  stores via PLC activation and  $\text{IP}_3$  generation followed secondarily by the sustained influx of extracellular  $\text{Ca}^{++}$  through SOCCs. The prevention of increases in  $[\text{Ca}^{++}]_i$  with various inhibitors of  $\text{Ca}^{++}$  signaling, which in and of themselves appear to have little effect on EGF-induced cellular proliferation, significantly inhibits EGF-induced mitogenesis. Finally, the manner whereby NSAIDs inhibit EGF-induced mitogenesis is very similar to other conditions in which EGF-induced SOCI is blocked ( $\text{Ca}^{++}$ -free media or the use of the SOCC blocker  $\text{La}^{+++}$ ). Thus the current study supports our premise that NSAIDs may attenuate colonic neoplasia by inhibiting EGF-induced  $\text{Ca}^{++}$  mobilization.

Caco-2 cells, derived from a human colonic carcinoma, were employed in the current study because these cells have been shown to possess EGF receptors<sup>20</sup> and have previously been shown to demonstrate physiologic responses to EGF.<sup>21</sup> Although these cells, when grown to postconfluency, have the unique ability to polarize, differentiate, and develop morphologic characteristics of normal enterocytes,<sup>22</sup> Caco-2 cells are more typical of colonic carcinoma cells early after plating. For these reasons, all experimentation was initiated when cells were 80% confluent. To date, we have not investigated the actions of EGF in other human colonic carcinoma cell lines.

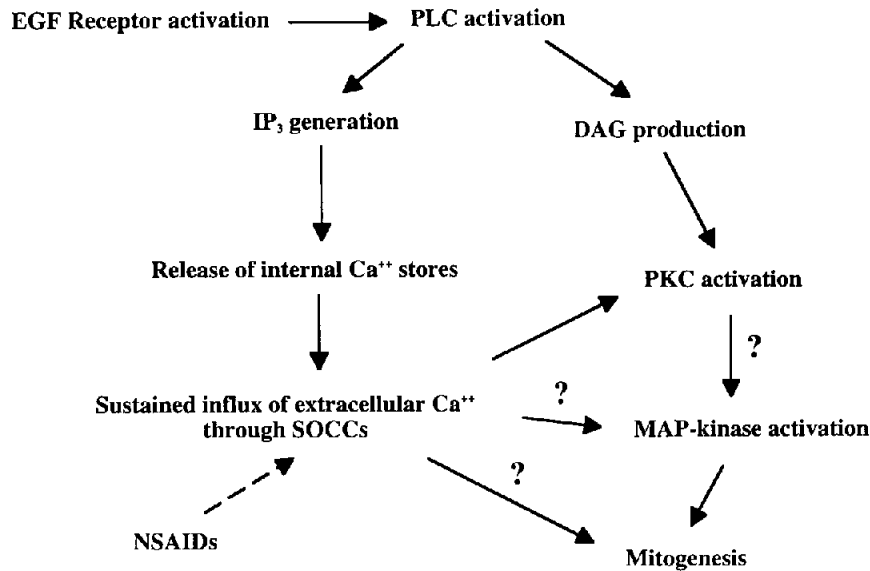
Our observations regarding the mechanism whereby EGF elicits changes in  $[\text{Ca}^{++}]_i$  are consistent with the literature. Several reports have suggested that EGF, after receptor binding, activates its tyrosine-specific protein kinase activity, which results in the subsequent phosphorylation of several substrates including PLC. Meisenhelder et al.<sup>23</sup> investigated the responses of both quiescent 3T3 mouse fibroblasts and A431 human epidermoid cells to EGF. They observed that EGF treatment resulted in the rapid phosphorylation of PLC tyrosines and serines and a subsequent increase in phosphatidylinositol turnover, suggesting that PLC is a substrate for EGF receptors. Prior work with mouse intestinal epithelial cells<sup>24</sup> and pancreatic AR42J<sup>25</sup> cells has demonstrated that the initial increase in  $[\text{Ca}^{++}]_i$ , as induced by EGF, was blocked by the PLC inhibitor U73122, but not the inactive agent U73343. These data confirm our observations and further suggest that EGF elicits an increase in PLC activity.

Following an initial increase in  $[\text{Ca}^{++}]_i$ , an effect that is likely the result of PLC stimulation followed by  $\text{IP}_3$  generation and the release of intracellular  $\text{Ca}^{++}$  stores, EGF elicits a prolonged phase of increased intracellular  $\text{Ca}^{++}$  levels that appears to involve the influx of extracellular  $\text{Ca}^{++}$  through voltage-independent  $\text{Ca}^{++}$  channels (or SOCCs). Magni et al.<sup>26</sup> reported that the EGF-induced sustained elevation in  $[\text{Ca}^{++}]_i$  in NIH-3T3 fibroblasts, was blocked by the imidazole derivative SC 38249, an SOCC blocker, in a manner very similar to cells tested after extracellular  $\text{Ca}^{++}$  was chelated with excess EGTA. Zhang et al.<sup>27</sup> also observed that the entry of extracellular  $\text{Ca}^{++}$  in hepatocytes, following EGF treatment, was not mediated by voltage-dependent  $\text{Ca}^{++}$  channels. In support of the current study, both Magni et al.<sup>26</sup> and Zhang et al.<sup>27</sup> also reported that the mitogenic effect of EGF in these two cell lines was inhibited when EGF-induced SOCI was prevented.

Previous work has also suggested that NSAIDs may inhibit  $\text{Ca}^{++}$  translocations involved with early cellular signaling. Abramson et al.<sup>28</sup> reported that indomethacin (30  $\mu\text{mol/L}$ ), piroxicam (50  $\mu\text{mol/L}$ ), or sodium salicylate (3 mmol/L) inhibited  $\text{Ca}^{++}$  uptake and increases in cytosolic  $\text{Ca}^{++}$  in human neutrophils stimulated by fMet-Leu-Phe through a process independent of any alteration of the affinity of fMet-Leu-Phe binding. Canesi et al.<sup>29</sup> investigated the EGF-activated signal transduction pathway in isolated digestive gland cells from mussels. They observed that indomethacin (20  $\mu\text{mol/L}$ ) blocked both EGF-induced  $\text{Ca}^{++}$  influx and subsequent mitogenesis.

Thus results from our laboratory and others suggest that  $\text{Ca}^{++}$  signaling, as induced by EGF, may play a role in stimulating quiescent (G0) cells to progress through G1 to DNA synthesis in S phase. Barbiero reported that BALB/c3T3 fibroblasts, following serum deprivation, exposed subsequently to fetal bovine serum demonstrated increases in  $[\text{Ca}^{++}]_i$ .<sup>30</sup> Furthermore, the addition of an extracellular  $\text{Ca}^{++}$  chelator (3 mmol/L EGTA) or the SOCC blocker SKF-96365A caused a significant reduction in fetal bovine serum-induced proliferation. Estacion et al.<sup>31</sup> observed that either low extracellular  $\text{Ca}^{++}$  or the SOCC blocker  $\text{La}^{+++}$ , both of which blocked platelet-derived growth factor (PDGF)-activated  $\text{Ca}^{++}$  influx, also inhibited PDGF-induced DNA synthesis.

Although there appears to be a linkage between elevations in  $[\text{Ca}^{++}]_i$  and mitogenesis, this mechanism is not well understood. One explanation may be that  $\text{Ca}^{++}$  influxes play an important role in activating mitogen-activated protein (MAP) kinases, which appear to be essential for the proliferative response of cells. Agents such as EGF, associated with proliferation,



**Fig. 7.** Proposed schematic of one mechanism whereby nonsteroidal anti-inflammatory drugs (NSAIDs) may inhibit mitogen-stimulated cellular proliferation (PLC = phospholipase C; DAG = diacylglycerol; IP<sub>3</sub> = inositol 1,4,5-trisphosphate; PKC = protein kinase C; SOCC = store-operated calcium channel; MAP kinase = mitogen-activated protein kinase).

produce short-lived increases in MAP kinases, whereas those that cause differentiation produce longer lived elevations, which are sustained for hours.<sup>32</sup> Kavanagh et al.<sup>33</sup> investigated the role of  $\text{Ca}^{++}$  fluxes in the activation of MAP kinases induced by low doses of ionizing radiation in A431 cells. They reported that the addition of either the intracellular  $\text{Ca}^{++}$  chelator BAPTA/AM, the  $\text{Ca}^{++}$  antagonist TMB-8, or the PLC inhibitor U73223 blocked radiation-induced  $\text{Ca}^{++}$  oscillations and inhibited MAP kinase stimulation. Interestingly, quiescent fibroblasts grown in nominal  $\text{Ca}^{++}$  (0.1 mmol/L) and then briefly exposed to 1 mmol/L  $\text{Ca}^{++}$ , in the absence of other protein growth factors, demonstrated MAP kinase activation in a manner quite similar to that of cells exposed to other mitogens such as EGF.<sup>34</sup> Protein kinase C may also play an important role in initiating the MAP kinase cascade. Diacylglycerol, generated as a product of PLC activity, in combination with an elevation in  $[\text{Ca}^{++}]_i$ , is known to activate various protein C kinase isoforms.<sup>35</sup> Soltoff<sup>36</sup> reported that both  $[\text{Ca}^{++}]_i$  elevations (via IP<sub>3</sub>) and diacylglycerol-activated MAP kinase in PC12 cells in a EGF receptor-dependent manner.

Despite numerous investigations, the exact mechanism(s) whereby EGF stimulates DNA synthesis in any cell type remains unknown. However, evolving data suggest that an early elevation in  $[\text{Ca}^{++}]_i$  may be a critical step in the cellular physiologic response characterized by a cascade initiated by substrate acti-

vation through the activation of kinases and phosphatases that link mitogenic receptor activation to the proliferative response. We propose that one mechanism whereby NSAIDs may ultimately attenuate colonic neoplasia is by blocking at least one step in this cascade—mitogen-induced  $\text{Ca}^{++}$  mobilization (Fig. 7).

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## Discussion

**Dr. B. Warner** (Cincinnati, Ohio). Why did you choose the Caco-2 cell line? As you know, it is a differentiated colon cancer cell line. Have you examined other cell lines? It seems that the tyrosine kinase cascade to phospholipase C is a generalized mechanism, which may begin with other

types of growth factors. Did you study growth factors other than EGF in terms of inhibition of proliferation? Finally, relevant to your overall hypothesis, you are suggesting that NSAIDs lessen metastasis or lessen the risk for cancer by inhibiting growth factor-induced proliferation. I am un-

aware of data to suggest that there is increased EGF receptor signaling in colon cancer or a correlation between EGF receptor quantity and colon cancer biology.

**Dr. F. Kokoska.** We investigated Caco-2 cells because they were well differentiated, and I think the less differentiated cell lines may be less responsive to growth hormones or mitogens. We have not investigated other cell lines. I want to stress that serum deprivation was initiated when cells were 80% confluent and that the cells were 2 to 3 days post confluent when the experiments ended. We wanted to work with the colorectal carcinoma side of Caco-2 so we did not allow cells to become post confluent 7 to 10 days, at which time they take on characteristics of small bowel epithelium. We have not looked at other growth factors. Many but not all other growth factors elicit calcium signaling similar to EGF, so it would be interesting to see if those that do not behave in a different manner. Finally, I think

that this is just one small piece of the puzzle with regard to the effect of NSAIDs on colon carcinogenesis. There are data suggesting that NSAIDs may be involved with apoptosis and other work looking at the immunomodulating effects of NSAIDs. But we think that cellular antiproliferation is one of the nonsteroidal effects.

**Dr. B. Bass** (Baltimore, Md.). I am interested in your choice of measurements of growth, namely, nucleic acids and proteins. Have you looked at the more conventional measures of growth such as cell counts and tritiated thymidine uptake?

**Dr. Kokoska.** I like to avoid radioactivity if I can, and I have found these assays to be extremely sensitive. Both assays were used previously to quantitate nucleic acids and proteins for gel loading, and I found them to be extremely sensitive and fairly easy to perform. I have not correlated them with other measures of proliferation.