Biochemical Pharmacology, Vol. 41, No. 6/7, pp. 1045–1054, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INFLUENCE OF 8-(*N*,*N*-DIETHYLAMINO)OCTYL-3,4,5-TRIMETHOXYBENZOATE (TMB-8) ON CELL CYCLE PROGRESSION AND PROLIFERATION OF CULTURED ARTERIAL SMOOTH MUSCLE CELLS

CLAUDE DESGRANGES,*† MICHEL CAMPAN,* ALAIN-PIERRE GADEAU,*
NATHALIE GUERINEAU,‡ PATRICE MOLLARD‡ and GABRIEL RAZAKA*

*Unité de Cardiologie INSERM U8, avenue du Haut-Lévêque, F-33600 Pessac, France; and
‡Laboratoire de Neurophysiologie, CNRS URA 1200, Université de Bordeaux 2, F-33076 Bordeaux Cedex, France

(*Received* 10 *September* 1990; *accepted* 7 *November* 1990)

Abstract—8-(*N*,*N*-Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), a putative inhibitor of intracellular calcium mobilization, causes a dose-dependent inhibition of serum-induced proliferation of arterial smooth muscle cells in culture. Neither early rise in cytosolic calcium concentration nor induction of early induced cell cycle dependent genes (c-fos, ornithine decarboxylase) are inhibited after serum stimulation in presence of 100 μM TMB-8. In contrast, expression of thymidine kinase, a gene normally induced in late-G_1 phase, is entirely inhibited by TMB-8. Taken together with flow cytometry studies, these results indicate that TMB-8 blocks cell cycle progression in mid- or late-G_1 phase by a mechanism not directly related to early responses to serum stimulation since TMB-8 is also effective when introduced several hours after serum stimulation.

Proliferation of intimal smooth muscle cells (SMCs) is a key event in development of atherosclerotic lesions [1, 2]. Many agonists have been shown to induce arterial SMC proliferation *in vitro* [for reviews 1, 3, 4]. In spite of their heterogeneity, these factors share several common mechanisms involved in the signal transduction into the cell, such as phosphatidylinositol hydrolysis, increase in intracellular cytosolic calcium and pH, activation of protein kinase C, calmodulin and other pathways that lead to DNA synthesis and cell proliferation by more or less defined mechanisms [for reviews 5, 6]. *In vitro*, the use of specific inhibitors has demonstrated the part of each of these pathways during the proliferative process. Indeed, inhibitors of receptor tyrosine kinase, of GTP-binding proteins, of Na^+–H^+ antiport, of protein kinase C, of calcium channels and of calmodulin decrease in different ways the proliferation of various cell types [7–9] including that of arterial SMC [10–14]. In addition, 8-(*N*,*N*-diethylamino)octyl - 3,4,5 - trimethoxybenzoate (TMB-8), a putative inhibitor of calcium release from sarcoplasmic reticulum in arterial SMCs [15–18] inhibits SMC DNA-synthesis induced by platelet-derived growth factor (PDGF) [17]. Despite its complex composition, serum is the reference substance to induce a complete progression through the cell cycle from quiescence exit to mitosis. Serum stimulation of arterial SMCs is generally accompanied by a transient rise in cytosolic calcium, resulting both from the release of stored intracellular calcium and from an influx of external calcium [19, 20]. The present study reports that TMB-8 inhibits serum-induced proliferation of cultured arterial SMCs essentially by blocking cell cycle progression in mid-G_1 phase. Although the inhibitory mechanisms of this product are not clearly defined, it appears that TMB-8 acts by a mechanism not directly related to the transient intracellular calcium increase induced after serum stimulation, since TMB-8 (i) does not inhibit this early serum-induced rise in calcium and (ii) continues to be active when introduced several hours after stimulation or in continuously cycling SMCs.

† Author to whom correspondence should be addressed at Unité 8 de l'INSERM, avenue du Haut-Lévêque, F-33600 Pessac, France.

MATERIALS AND METHODS

Cell culture. Arterial SMCs were isolated from thoracic and abdominal aortas of male Wistar rats by enzymatic dissociation. Aortas were removed aseptically and cleaned of adventitia, and after cutting into small fragments, were incubated in 10 mL Dulbecco modified Eagle's Medium (DMEM) containing 1 mg/mL collagenase and 0.1 mg/mL elastase for successive 30 min periods at 37° in a stirring flask. Single cell suspensions were separated from undigested tissues and centrifuged (10 min, 200 *g*) after addition of 10% fetal calf serum (FCS). Cell pellets were resuspended in DMEM supplemented with 10% FCS and seeded into 25 cm^2 flasks. The medium was changed every 2 days and confluency was reached about 10 days after seeding. Secondary cultures were obtained from these primary cultures by serial passages after harvesting the cells with trypsin–EDTA. These cells were stained by L1, a monoclonal antibody that recognizes a surface antigen specifically expressed in cultured rat arterial SMCs [21]; furthermore, they were stained by 1A4, a monoclonal antibody recognizing exclusively α-smooth muscle actin [22], suggesting the smooth

muscle nature of these cells. Cells with identical growth characteristics from passages 5 to 20 were used for the following experiments, at cell densities varying from 2 to 8×10^4 cells/cm^2. Quiescent cultures were obtained by a 2 day incubation of cells in a serum free medium; in these conditions SMCs were essentially blocked in G_0/G_1 phase as demonstrated by flow cytometry and by the absence of DNA synthesis. Cell cycle progression was induced by addition of fresh medium containing 10% FCS. S-phase arrested SMCs were obtained by a 20 hr treatment in 1 mM hydroxyurea containing medium. SMCs were synchronized in metaphase by 8 hr demecolcine (0.1 μg/mL) treatment 18 hr after serum stimulation; mitotic cells were detached by shaking, then reseeded in fresh medium.

Thymidine kinase assay. After washing in serum-free DMEM, cells were lysed by sonication in 50 mM Tris–HCl (pH 8), 5 mM β-mercaptoethanol in 50% glycerol. Cell extract (25 μL) were mixed with 75 μL of a solution containing 5 mM phosphoenolpyruvate, 0.125 mg/mL pyruvate kinase, 5 mM ATP, 10 mM NaF, 5 mM $MgCl_2$, 25 mM Tris–HCl (pH 8) and 10 μCi [*methyl*-^{3}H]thymidine. After incubation at 37° for 10 and 20 min, 10 μL of the reaction mix was spotted onto DE 81 filters (Whatman) and immediately air dried. After successive washes with 10 mM ammonium formate, H_2O and ethanol, filters were dried in ether and placed in scintillation vial containing 0.2 mL H_2O and 1 mL Soluene 350 (Packard) and counted for radioactivity in 10 mL of scintillation cocktail (OCS, Amersham). Enzyme activity was expressed as pmol of thymidine converted into thymidylate per minute by an extract corresponding to 10^6 cells at 37°.

Ornithine decarboxylase assay. After washing in PBS, SMCs were lysed by sonication in 50 mM Tris–HCl (pH 7.2) containing 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, 10 mM dithiothreitol, and centrifuged at 12,000 *g* for 15 min. ODC activity was determined by measuring the release of radioactive CO_2 from L-[1-^{14}C]ornithine as described previously [23] with some modifications: 100 μL cell extract were incubated with 10 μL L[1-^{14}C]ornithine in a conical tube (Falcon 2095) closed with a rubber stopper carrying a suspended center well (Kontess) containing 100 μL Soluene 350. After 1 hr incubation, the reaction was stopped by addition of 0.3 mL of 10% trichloracetic acid. After a further 90 min period the generated CO_2 trapped by the quaternary amine of soluene was determined by counting the radioactivity of the center well in 10 mL OCS. The ODC activity was expressed as pmol of radioactive CO_2 released per hour by extracts corresponding to 10^6 cells.

Protein synthesis. Total protein synthesis was measured by adding 1 μCi/mL of L-[4,5-^{3}H]leucine to the culture during different times according to the experiment. After washing in PBS, cells were lysed and proteins were precipitated by 0.5 M perchloric acid treatment; after centrifugation the protein pellet was dissolved in 0.5 M NaOH and counted in PCS scintillation fluid. Protein synthesis was estimated as nmol of radiolabelled leucine incorporated into proteins per 10^6 cells and during the time of incubation.

Monitoring of cytosolic free calcium concentration in individual arterial SMCs. SMCs were grown on glass coverslides for 2 days in serum-free DMEM. Cytosolic calcium concentration $[Ca^{2+}]_i$ was determined in individual cells by dual emission microfluorimetry using indo 1 as intracellular calcium probe [24]. Before the dye loading with indo 1, the nutrient medium was replaced with a modified Hank's solution containing 142.6 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES and buffered to pH 7.3 with NaOH. The cells were loaded with indo 1 by exposure to 5 μM indo 1 pento-acetomethylester (indo 1/AM) and 0.02% Pluronic F-127 in Hank's solution for 30 min at 20°. $[Ca^{2+}]_i$ was estimated in individual cells from indo 1 fluorescence by the ratio method using single wavelength excitation (355 nm) and dual emission (405 and 480 nm) exactly as previously described [25]. Microfluorimetric experiments were carried out at 37°. Test compounds were applied during those experiments by low pressure ejection from micropipettes (tip diameter, 2–5 μm) positioned close to the recorded cell (within 10–30 μm).

Flow cytometry. Progression through the cell cycle was monitored by flow cytofluorometry. After trypsin harvesting, cells were washed in serum-free DMEM and resuspended in this medium at 10^6 cells per mL. These cells were stained by acridine orange according to Darzynkiewicz *et al.* [26] and analysed for DNA and RNA content by using a cell sorter (ATC 3000-ODAM).

RNA preparation and hybridization. Total RNAs were prepared either according to Cathala *et al.* [27] after cell lysis by guanidinium isothiocyanate or by the Hatch technique [28]. After denaturation, RNAs were electrophoresed in 1% agarose gel containing 2.2 M formaldehyde [29], transferred by blotting to nylon membranes (Hybond N, Amersham) and covalently fixed by exposure to 260 nm UV source. After prehybridization at 42° [29] membranes were hybridized 2 days at 42° in the same buffer containing purified insert DNA from cloned c-fos [30], ODC (derived from pmODC-1) [31] and TK [32], labelled after oligo-priming with [α-^{32}P]dCTP. After washing, hybridized filters were autoradiographed using Kodak intensifying screens and Kodak X-OMAT AR films.

Materials. Materials were obtained from the following sources: DMEM, PBS, serum and trypsin–EDTA from GIBCO-BRL Life Technologies (Uxbridge, U.K.); NA-agarose from Pharmacia (Uppsala, Sweden); acridine orange from Polyscience Inc. (Warrington, PA); Elastase (porcine pancreas) from Serva (Heidelberg, F.R.G.); TMB-8, demecolcine, hydroxyurea, collagenase (type IV, *Clostridium histolyticum*) and anti-α-actin from the Sigma Chemical Co. (St Louis, MO); phosphoenolpyruvate, and indo 1/AM from Calbiochem (La Jolla, CA); pyruvate kinase and ATP from Boehringer (Mannheim, F.R.G.); pyridoxal phosphate from Fluka (Buchs, Switzerland); Pluronic F-127 from Molecular Probes (Eugene, OR).

Radiolabelled compounds used in this study were: [*methyl*-^{3}H]thymidine (52 Ci/mmol) and L-[4,5-^{3}H]leucine (139 Ci/mmol) from Amersham; L-[1-^{14}C]ornithine (50 mCi/mmol) from CEA-France;

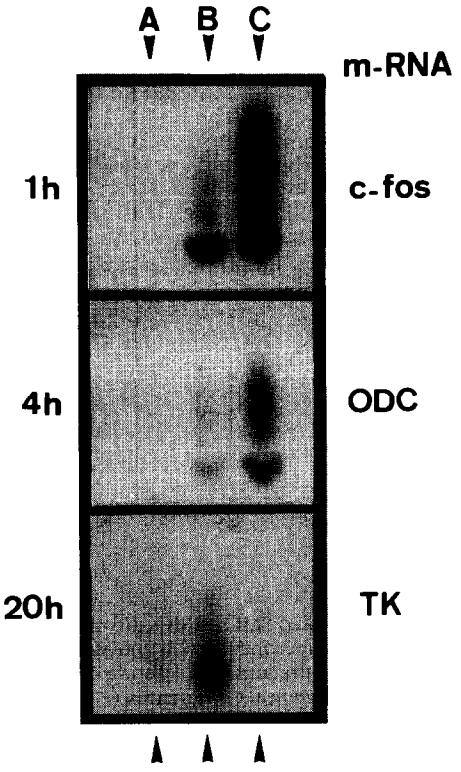

Fig. 1. Influence of TMB-8 on induction of cell cycle dependent genes in serum stimulated arterial SMCs. Quiescent SMCs are stimulated by 10% FCS-DMEM with or without 80 μM TMB-8. Cells are harvested at the indicated times after serum addition; RNAs extracted from 10^6 cells are used for Northern blot hybridization as described in Materials and Methods, respectively with c-fos, ODC and TK random primed probes, to detect specific mRNAs. (A) unstimulated cells. (B) serum-stimulated cells. (C) serum-stimulated cells in presence of 80 μM TMB-8.

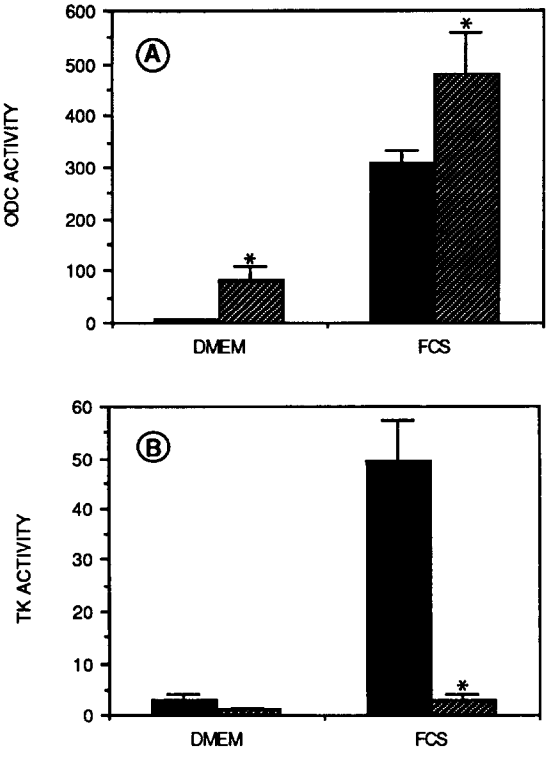

Fig. 2. Effects of TMB-8 on induction of ODC and TK activities after serum-stimulation of quiescent arterial SMCs. ODC (A) and TK (B) activities are determined on three different flasks as described in Materials and Methods, 4 and 20 hr respectively after medium change with serum-free medium (DMEM) or 10% FCS-DMEM (FCS): in absence (black bars) or in presence (hatched bars) of 80 μM TMB-8. Results are expressed as mean ± SD. Statistically significant differences ($P < 0.05$) between TMB-8 treated and control cells are indicated by *.

[α-^{32}P]dCTP (3000 Ci/mmol) from NEN-Du Pont de Nemours.

Statistics. Statistical data are means ± SD and were analysed by Student's *t*-test; $P < 0.05$ was considered significant.

RESULTS

Serum stimulation of quiescent SMCs

Several responses occur after FCS stimulation of quiescent serum deprived arterial SMCs. Among them, some cell cycle dependent genes which are not or faintly expressed in quiescent SMCs, are chronologically induced after serum stimulation. Figure 1 shows that c-fos, ODC and TK mRNAs which are not present in quiescent SMCs (row A), are maximally expressed respectively 1, 4 and 20 hr after serum stimulation (row B). In the same time, ODC and TK activities increase significantly after serum addition in comparison to those of unstimulated SMCs (Fig. 2A and B). At last, DNA synthesis begins 10–12 hr after stimulation (not shown) and the first doubling in cell number requires about 24–28 hr; doubling time of exponentially growing SMCs in 10% FCS-DMEM is about 20–22 hr. Cells used in this study do not show significant variations in cell cycle dependent gene induction and in growth characteristics.

TMB-8 effects on SMC proliferation and cell cycle progression

Addition of TMB-8 at the time of serum stimulation of quiescent SMCs prevents cell proliferation in a concentration dependent manner, with an IC_{50} of about 30 μM; 80 μM TMB-8 completely inhibits the appearance of mitosis (Fig. 3). Preincubation with TMB-8 before serum addition undergoes to a similar inhibition (data not shown). TK mRNAs fail to appear in SMCs stimulated in presence of TMB-8, while c-fos and ODC mRNAs are found in higher quantities than in control SMCs stimulated by serum without TMB-8 (Fig. 1, rows B and C). Similarly, the enhancement of TK activity by serum is completely suppressed in presence of TMB-8, while ODC activity is found higher than in control (Fig. 2 A and B). Flow cytometry studies show that, 18 hr after serum stimulation, 93% of SMCs remain in G_1 in presence of 80 μM TMB-8, while in the

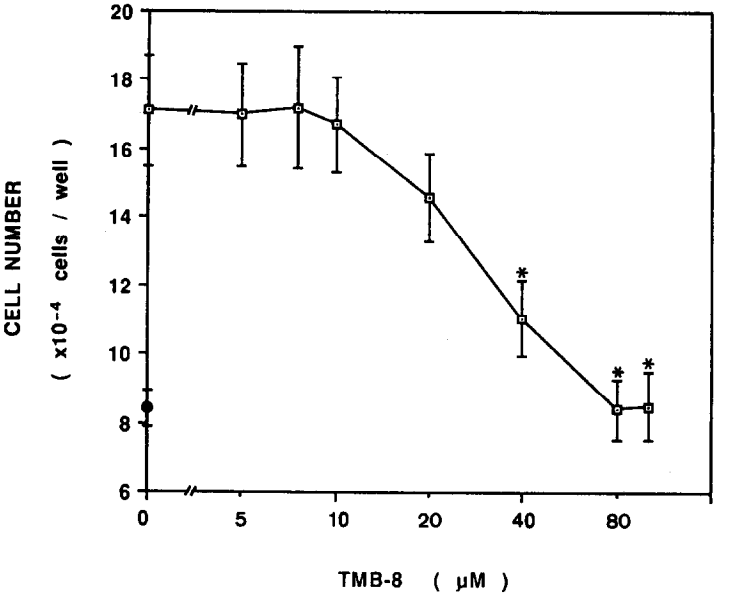

Fig. 3. Effect of TMB-8 on serum-induced proliferation of growth arrested SMCs. SMCs are plated in 24-well culture plates and made quiescent by serum deprivation as described in Materials and Methods. At the time of serum stimulation. TMB-8 is added at different concentrations and cells are counted 30 hr later (4 wells/point). Values are the mean ± SD. Statistically significant differences ($P < 0.05$) between TMB-8 treated and control cells are shown (*).

Table 1. Effects of TMB-8 on cell cycle progression: flow cytometry studies

Cells	Treatment	% of cells in G1A	G1B	S	G2 + M
(A) Quiescent SMCs	none	80	15	3	2
	FCS	8	25	30	37
	FCS + TMB-8	35	58	4	3
(B) Cycling SMCs	FCS	35	34	14	17
	FCS + TMB-8	47	37	11	5

(A) Quiescent arterial SMCs are stimulated by 10% FCS for 18 hr without or with 80 μM TMB-8 and analyzed by flow cytometry as described in Materials and Methods. The number of cells distributed in each predetermined window corresponding to early-G1 phase (G1A, low RNA content), to mid- or late-G1 phases (G1B, high RNA content), to S and G2 + M phases, are automatically determined on a total number of 20,000 cells. Results are expressed as the percentage of cells found in each window vs the total number of analysed cells.

(B) Culture media of asynchronously growing SMCs are changed with fresh media containing or not 80 μM TMB-8. Twenty-one hours later, repartition of SMCs throughout the cell cycle is analysed as described above.

same time 67% of cells have passed through the G_1/S boundary in absence of TMB-8 (Table 1). Furthermore, in cells treated for 18 hr with TMB-8, TK activity begins to increase 6 hr after the removal of TMB-8 from the culture medium, while in G_0 quiescent SMCs TK activity only increases 9 hr after serum stimulation (not shown). Taken together, these results provide evidence that TMB-8 may block cell cycle progression in mid-G_1 phase when introduced prior to or at the same time as serum.

Effect of time addition of TMB-8 on proliferative response

As shown in Fig. 4 (A and B), accumulations of c-fos and ODC mRNAs in SMCs occur when TMB-8 is applied together with serum as well as when it is added at different times after the serum stimulation. c-fos and ODC mRNA contents are highest when TMB-8 is introduced during the first 30 min (for c-fos) and the first hour (for ODC) after stimulation; then the superaccumulation of mRNAs decreases

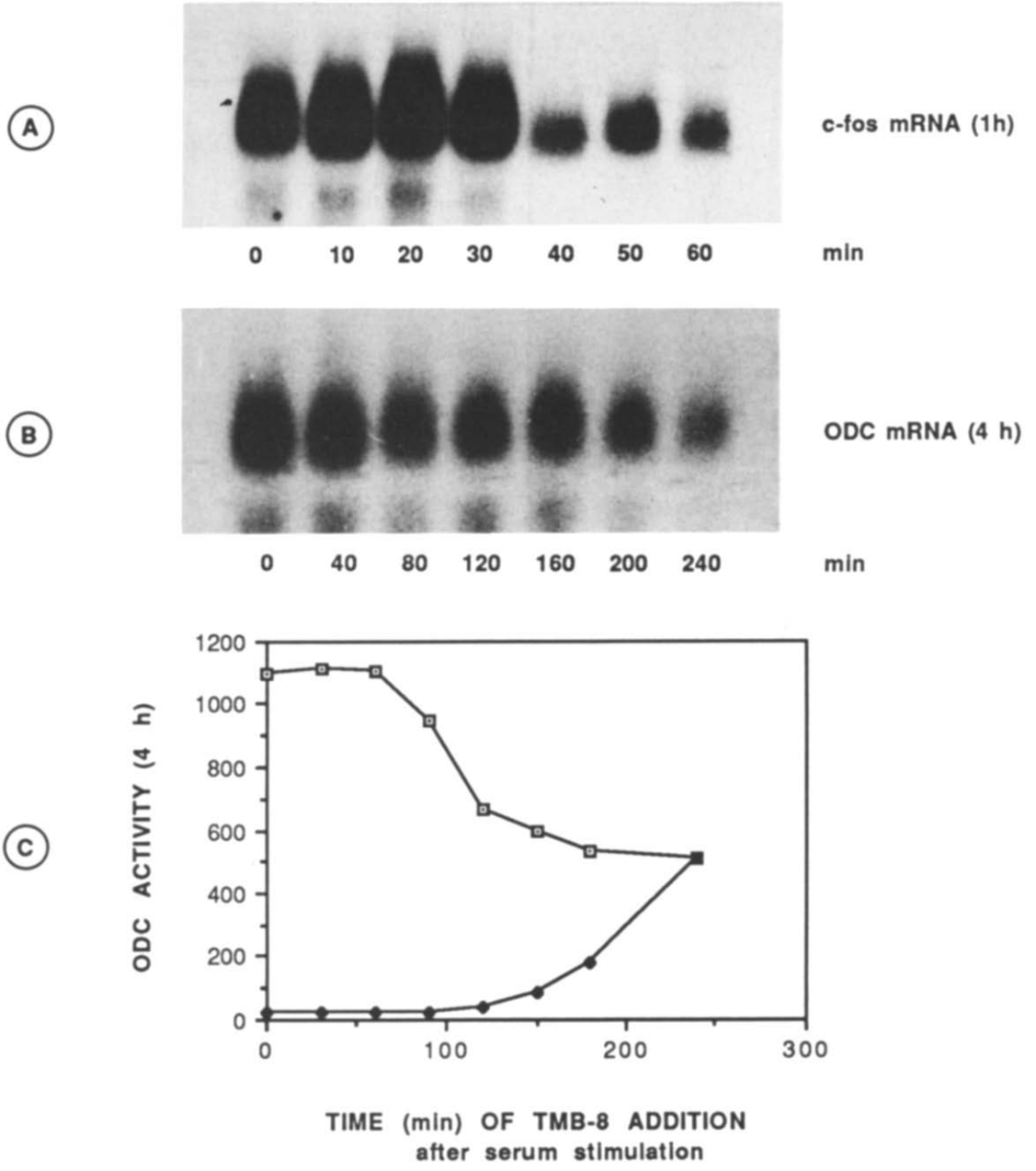

Fig. 4. Addition of TMB-8 at different times after serum stimulation of quiescent SMCs: effects on c-fos and ODC gene expression. 80 μM TMB-8 (□) is added at different times after serum stimulation of quiescent SMCs. (A) and (B) RNAs are extracted 1 and 4 hr after serum addition for respective determination of c-fos and ODC mRNAs as described previously. (C) acellular extracts are prepared 4 hr after stimulation and ODC activity is determined as described in Materials and Methods. Influence of time addition of actinomycin D (1 μg/mL) is processed in parallel (♦). (A), (B) and (C) experiments have been performed in parallel with the same batch of cells.

with the time of TMB-8 addition to come back to maximal control levels (respectively 60 and 240 min for c-fos and ODC). ODC activity shows exactly the same behaviour as ODC-mRNA content according to the time of TMB-8 addition after serum stimulation (Fig. 4 C); in comparison, actinomycin D, a transcription inhibitor, strongly decreases ODC activity appearance. The decrease of ODC activity observed after the 4th hr following serum stimulation is not modified when TMB-8 is introduced at that time (not shown).

In contrast to c-fos and ODC genes, the induction of TK expression is inhibited not only when 80 μM TMB-8 is present at the time of serum stimulation, but also when this agent is introduced later (0 to 8.5 hr) (Fig. 5). When TMB-8 is added from 8.5 to

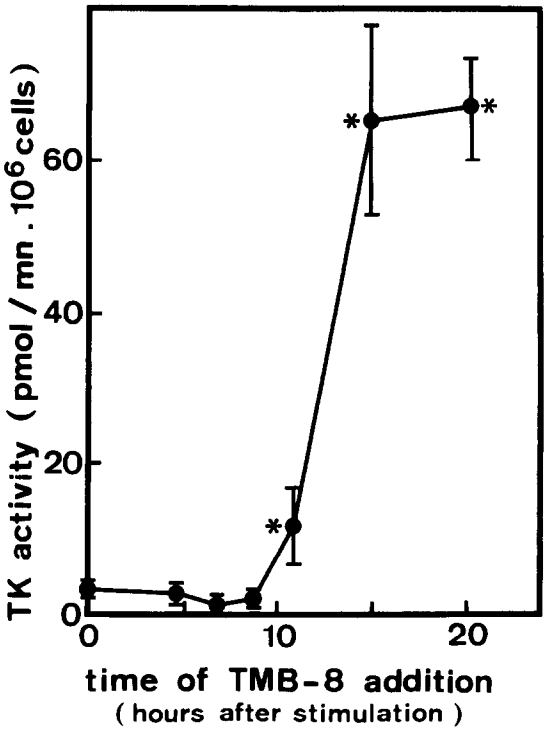

Fig. 5. Addition of TMB-8 at different times after serum stimulation of quiescent SMCs: effect on TK induction. 80 μM is added at the indicated time points after stimulation of quiescent SMCs and the incubation is completed up to 20 hr. At that time, cells are treated as previously described for TK determination. Values are the mean ± SD of three different flasks. Significant differences ($P < 0.05$) between cells treated by TMB-8 at the time of stimulation and cells treated at different times after stimulation are indicated (*).

15 hr after stimulation, TK induction is only partially inhibited. After that time, no marked effect on TK activity is detected.

When TMB-8 is added during the first 8–10 hr following serum stimulation, SMCs do not cross the G_1/S boundary and accumulate in G_1 phase as demonstrated by inhibition of TK appearance (Fig. 5) and flow cytometry studies (Table 1 and not shown). Introduction of TMB-8 after that time demonstrates that TMB-8 only slightly decreases the rate of SMC divisions during the first cycle. When TMB-8 is added after hydroxyurea removal, in S-phase synchronized SMCs (see Materials and Methods), proliferation is faintly decreased, while proliferation is not disturbed when TMB-8 is added, after demecolcin removal, in M-phase synchronized SMCs. Moreover, the end of mitosis is not inhibited when anaphase blocked SMCs, obtained by demecolcine treatment, are reseeded in presence of TMB-8 (not shown).

Finally, when 80 μM TMB-8 is added on exponentially growing SMCs the cell number continues to increase for 24 hr but at a lower extent than in the control group, then stabilizes. Although flow cytometry studies show that the percentage of cells in S and G_2-M phases is faintly higher in TMB-8 treated cycling SMCs than in quiescent stimulated SMCs, suggesting a moderate effect of TMB-8 during the last part of the cell cycle, above all these studies demonstrate that TMB-8 treated SMCs preferentially accumulate in G_1 phase (Table 1).

Effects of TMB-8 on serum-induced $[Ca^{2+}]_i$ *rise*

The results described above address the question whether TMB-8 blocks cell proliferation in reducing the $[Ca^{2+}]_i$ response to this growth factor cocktail. Therefore, $[Ca^{2+}]_i$ has been monitored in individual SMCs loaded with indo 1 as a fluorescent calcium probe. All these $[Ca^{2+}]_i$ measurements have been performed on cell cultures in which TMB-8 is fully active in altering cell proliferation as described above.

At rest, quiescent G_0/G_1 SMCs show a basal $[Ca^{2+}]_i$ level of 97 ± 23 nM (N = 52). Short application of 10% FCS induces a single massive rise in $[Ca^{2+}]_i$ (peak value 369 ± 80 nM, N = 18) (Fig. 6A). Thereafter $[Ca^{2+}]_i$ returns to the basal level within 30–60 sec. As previously shown in $[Ca^{2+}]_i$ measurements on cell populations [19], our single cell $[Ca^{2+}]_i$ recordings strengthen the suggestion that the initial $[Ca^{2+}]_i$ transient triggered by serum is mainly due to a release of calcium ions from internal stores, since serum evokes a nearly similar response in the absence of external calcium (peak value 349 ± 62 nM, N = 20) (Fig. 6B). In these conditions, it is demonstrated that TMB-8 does not abolish acute $[Ca^{2+}]_i$ response to serum at concentrations effective in altering serum-dependent proliferation. Indeed, FCS and TMB-8 applied together evoke $[Ca^{2+}]_i$ transients (peak value 421 ± 66 nM, N = 12) (Fig. 6C) similar to those induced by FCS alone, whether calcium is present (peak value 421 ± 66 nM, N = 12) (Fig. 6C) or not (peak value 362 ± 32 nM, N = 5) (Fig. 6D) in the bathing solution. Furthermore, 100 μM TMB-8 pretreatment from 5 to 60 min is also ineffective in reducing $[Ca^{2+}]_i$ responses to serum from cells bathed either in the presence (peak value 427 ± 93 nM, N = 9) (Fig. 6E) or in the absence of external calcium (peak value 382 ± 53 nM, N = 53) (Fig. 6F).

Influence of TMB-8 on protein synthesis

Protein synthesis, estimated by continuous incubation with labelled leucine, is not significantly affected by TMB-8 during the first 4 hr following serum stimulation of quiescent SMCs; in contrast, an important decrease in leucine incorporation (up to 40%) is detected after a 20 hr incubation in presence of TMB-8. One hour pulse experiments, realized at different times after stimulation, demonstrate that the difference in protein synthesis between TMB-8 treated and control cells become significant 5 hr after stimulation (Fig. 7). However, protein synthesis is not entirely inhibited as with cycloheximide but remains constant for several hours, suggesting that the apparent inhibition of protein synthesis observed when SMCs are stimulated in presence of TMB-8, is probably a consequence of progression blockade in a point of G_1 phase where the protein synthesis is relatively low in comparison to that of untreated cells which has reached S and G_2 phases where protein synthesis is higher.

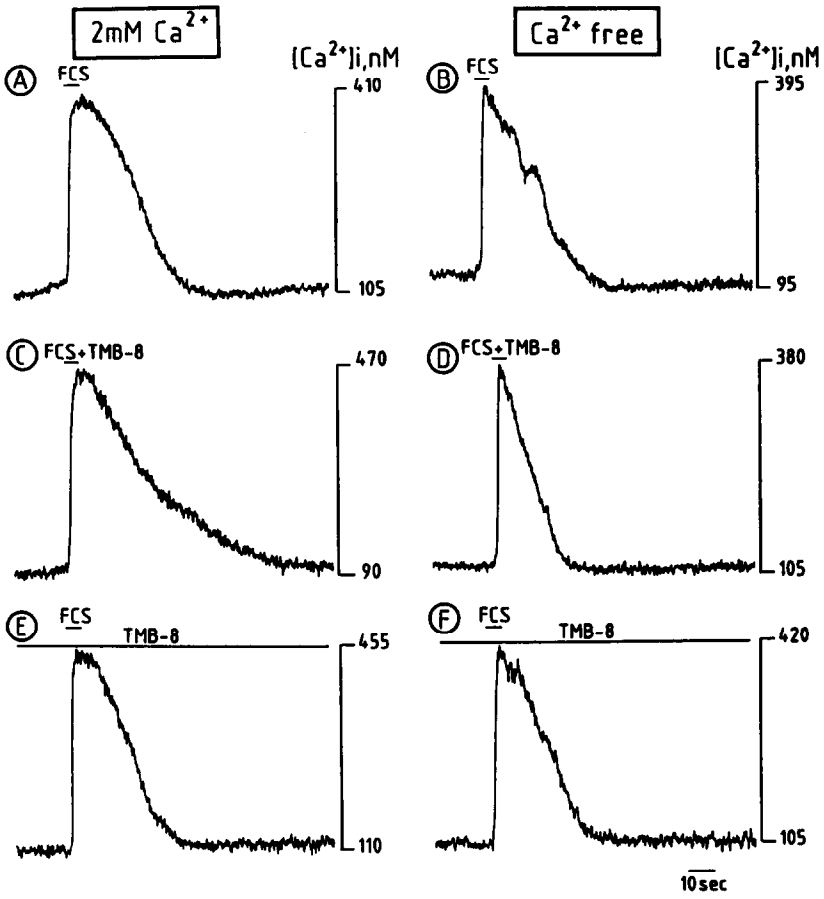

Fig. 6. Effects of TMB-8 on serum-evoked $[Ca^{2+}]_i$ rise in arterial SMCs. $[Ca^{2+}]_i$ is monitored in individual SMCs loaded with the fluorescent calcium probe indo 1 (see Materials and Methods). Cells are bathed in a saline solution containing (panels A, C, E) or not (panels B, D, F) calcium ions. In the latter, EGTA is added at 2 mM to chelate residual calcium ions. (A) and (B) FCS (10% in saline solution) is applied from a "puffer" pipette, (C) and (D) FCS (10%) and TMB-8 (100 μM) are applied together, (E) and (F) FCS (10%) is applied onto cells pretreated during 10 min with TMB-8 (100 μM).

DISCUSSION

The present results show that TMB-8 dose dependently inhibits proliferation of arterial SMCs grown in culture. TMB-8 exerts its inhibitory action in quiescent serum-stimulated as well as continuously cycling SMCs in culture. Although it has been previously shown that a preincubation with TMB-8 dramatically inhibited thymidine incorporation (S phase entry) in SMCs stimulated by PDGF [15, 17], our results localise the action of TMB-8 in the cell cycle of serum-stimulated SMCs. TMB-8 operates essentially during the G_1 phase of the cell cycle. Indeed, when applied prior or during serum stimulation of G_0 arrested SMCs, TMB-8 acts by blocking cell cycle progression in the G_1-phase between the induction of competence genes (c-fos, ODC) and the activation of TK gene (end of G1 phase). This blockade in mid-G_1 phase is confirmed by cytofluorometric studies (Table 1).

TMB-8 has been previously described as a blocker of calcium release from sarcoplasmic reticulum in both skeletal and smooth muscle cells [33]. Therefore, it would be speculated that TMB-8 might act on cell proliferation through an alteration of calcium homeostasis. However, our results demonstrate that TMB-8 is ineffective in altering serum-evoked transient calcium increase due to calcium mobilization in cells previously blocked in G_0-G_1 phase, whereas TMB-8 is fully active in blocking cell proliferation in the same cell cultures. They are in disagreement with data reported previously in which TMB-8 has been found to inhibit both calcium response and cell proliferation evoked by PDGF [15, 17] as well as cytosolic calcium rises triggered by other stimuli [16–18]. The lack of TMB-8 effects on calcium stores might be linked to the fact that TMB-8 sensitive stores represent only a few percentage of total calcium stores involved in the serum-evoked calcium response from SMCs used in this study. Indeed, it has been previously shown in SMCs that TMB-8 had no effect on cytosolic calcium rises induced by other stimuli such as vasopressin [34] or pH increase [18]. Likewise, in other cell types, TMB-8 did not exert significant effect on angiotensin-induced calcium release in adrenal glomerulosa cells [35], thrombin-evoked calcium transient in human platelets even in absence of extracellular calcium [36] and IP_3-

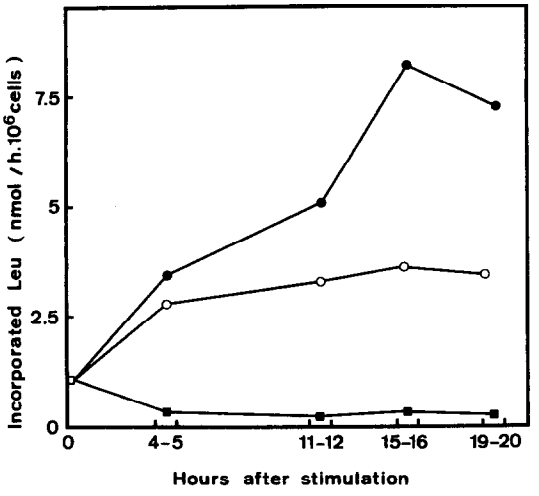

Fig. 7. Influence of TMB-8 on protein synthesis after serum stimulation of quiescent SMCs. Quiescent SMCs are stimulated by 10% FCS with (○) or without (●) 80 μM TMB-8 or 1.5 μg/mL cycloheximide (■). At indicated times, tritiated leucine is added for 1 hr to the culture medium. Then, cells are treated as described in Materials and Methods for determination of tritiated leucine incorporated into newly synthesized proteins. Each data point represents the mean of two different experiments (deviations lower than 15%).

induced calcium release in permeabilized cells [37]. Furthermore, recent data using calcium imaging technology provided evidence that distinct internal calcium stores may be involved in response to different stimuli [38]. As serum is a mixture of hormones and growth factors, it would trigger calcium release from several stores, some of them TMB-8 insensitive. It will be of interest to characterize the calcium responses to the different serum components and their respective sensitivity to TMB-8. Furthermore, Pian-Smith *et al.* [39] had shown that TMB-8 increased cytosolic calcium concentration in pancreatic B-cells whether calcium was present or not in the bathing solution suggesting that calcium release from internal stores was involved in response to TMB-8. Similarly, treatment of arterial SMCs by high concentrations (above 100 μM) of TMB-8 increased the basal $[Ca^{2+}]_i$ and reduced the size of the ionomycine sensitive calcium pool [16]. From these results, it may be concluded that TMB-8 action on calcium movements differs according to the cellular type and the nature of the agonist.

Our results also demonstrate that TMB-8 blocks TK induction on late-G_1 phase and further cell cycle progression whether this drug is applied simultaneously with serum or 8 hr later. This strengthens the hypothesis that TMB-8 decreases serum cell proliferation by acting independently of the earliest serum-evoked mechanisms, such as the initial transient cytosolic calcium response. However, a direct effect of TMB-8 on subsequent events may not be excluded. In this context, recent studies have demonstrated that competence growth factors induced a rapid and sustained rise in $[Ca^{2+}]_i$ in primed competent cells [40, 41]. Therefore, TMB-8 may act on intracellular calcium movements necessary for further progression through the G_1 phase and/or other calcium independent mechanisms.

Indeed, as for TMB-8, the inhibitory action of some other previously described inhibitors of arterial SMC proliferation is restricted to the first hours of progression through the G_1 phase: these include prostaglandins [42, 43], c-AMP [43], atrial natriuretic peptide and sodium nitroprusside [44], heparine [45], phorbol esters [46, 47], staurosporine [14], γ-interferon [48]. If the inhibitory effects of heparin or γ-interferon are not clearly understood, in contrast the role of different other inhibitors might be associated with actions on various intracellular messenger systems. For instance, an increase of intracellular c-AMP concentrations is probably directly implicated in the inhibition of cell cycle progression in G_1 phase generated by prostaglandins [42, 43, 49] and probably by other c-AMP elevating agents such as adenosine [50] or catecholamines [51]. Identically, c-GMP accumulation in SMCs induced by c-GMP elevating vasodilatators certainly plays a part in SMC growth inhibition [44, 52]. In addition, protein kinase C may serve as positive [14] or negative [46, 47] regulator of mid- or late-G_1 phase progression. Concerning the TMB-8 action, further investigations are necessary to examine if activation or inhibition of protein kinases A or C or of other intracellular messenger systems may be related to inhibition of arterial SMC proliferation in advanced G_1 phase. Therefore, these studies may help to identify late-G_1 targets for other growth inhibitors of arterial SMCs and to better define their role as potential modulators of SMC proliferation for future antiatherosclerotic therapy.

Acknowledgements—This investigation was supported by grants from INSERM (U8 and 874003), Squibb Laboratories (86074), University of Bordeaux 2 (6245), EPR (8903036), CNRS (URA 1200) and LNCC. A.P.G. and M.C. are respective recipients of fellowships from Squibb Laboratories and GRRC. The authors thank all the providers of molecular probes used in this study, Dr F. Belloc for his precious help in flow cytometry studies, F. Dupuch and I. Belloc for their expert technical assistance and M. Faucher for her excellent editorial assistance.

REFERENCES

1. Ross R, The pathogenesis of atherosclerosis—An update. *N Engl J Med* **314**: 488–500, 1986.
2. Munro JM and Cotran RS, The pathogenesis of atherosclerosis: Atherogenesis and Inflammation. *Lab Investig* **58**: 249–261, 1988.
3. Nilsson J, Growth factors and the pathogenesis of atherosclerosis. *Atherosclerosis* **62**: 185–199, 1986.
4. Schwartz SM, Campbell GR and Campbell JH, Replication of smooth muscle cells in vascular disease. *Circ Res* **58**: 427–444, 1986.
5. Rozengurt E, Early signals in the mitogenic response. *Science* **234**: 161–166, 1986.
6. Olashaw NE and Pledger WJ, Cellular mechanisms regulating proliferation. In: *Advances in Second Messenger and Phosphoprotein Research* (Eds. Greengard P and Robinson GA, Vol. 22, pp. 139–173. Raven Press, New York, 1988.

7. Besterman JM, Tyrey SJ, Cragoe EJ and Cuatrecasas P, Inhibition of epidermal growth factor-induced mitogenesis by amiloride and an analog: Evidence against a requirement for Na^+/H^+ exchange. *Proc Natl Acad Sci USA* **81**: 6762–6766, 1984.
8. Chambard JC, Paris S, L'Allemain G and Pouysségur J, Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature* **326**: 800–803, 1987.
9. Yaish P, Gazit A, Gilon C and Levitzki A, Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science* **242**: 933–935, 1988.
10. Thyberg J and Palmberg L, The calcium antagonist nisoldipine and the calmodulin antagonist W-7 synergistically inhibit initiation of DNA synthesis in cultured arterial smooth muscle cells. *Biol Cell* **60**: 125–132, 1987.
11. Tomita M, Hirata Y, Takata S and Fujita T, Effects of calcium-antagonists and calmodulin inhibitors on DNA synthesis in cultured rat vascular smooth muscle cells. *Endocrinology* **34**: 313–318, 1987.
12. Hultgardh-Nilsson A, Nilsson J, Jonzon B and Dalsgaard GJ, Coupling between inositol phosphate formation and DNA synthesis in smooth muscle cells stimulated with neurokinin A. *J Cell Physiol* **137**: 141–145, 1988.
13. Takagi Y, Hirata Y, Takata S, Yoshimi H, Fukuda Y, Fujita T and Hidaka H, Effects of protein kinase inhibitors on growth factor-stimulated DNA synthesis in cultured rat vascular smooth muscle cells. *Atherosclerosis* **74**: 227–230, 1988.
14. Matsumoto H and Sasaki Y, Staurosporine, a protein kinase C inhibitor interferes with proliferation of arterial smooth muscle cells. *Biochem Biophys Res Commun* **158**: 105–109, 1989.
15. Herman B, Roe MW, Harris C, Wray B and Clemmons D, Platelet-derived growth factor-induced alterations in vinculin distribution in porcine vascular smooth muscle cells. *Cell Motility Cytoskel* **8**: 91–105, 1987.
16. Tawada Y, Furukawa KI and Shigekawa M, ATP-induced calcium transient in cultured rat aortic smooth muscle cells. *J Biochem* **102**: 1499–1509, 1987.
17. Roe MW, Hepler JR, Harden TK and Herman B, Platelet-derived growth factor and angiotensin II cause increases in cytosolic free calcium by different mechanisms in vascular smooth muscle cells. *J Cell Physiol* **139**: 100–108, 1989.
18. Siskind MS, McCoy CE, Chobanian A and Schwartz JH, Regulation of intracellular calcium by cell pH in vascular smooth muscle cells. *Am J Physiol* **256**: C234–C240, 1989.
19. Kariya K and Takai Y, Distinct functions of down-regulation-sensitive and -resistant types of protein kinase C in rabbit aortic smooth muscle cells. *FEBS Lett* **219**: 119–124, 1987.
20. Kariya K, Kawahara Y, Fukuzaki H, Hagiwara M, Hidaka H, Fukumoto Y and Takai Y, Two types of protein kinase C with different functions in cultured rabbit aortic smooth muscle cells. *Biochem Biophys Res Commun* **161**: 1020–1027, 1989.
21. Printseva OYu, Faerman AI and Tjurmin AV, The expression of specific surface antigen of smooth muscle cells is related to proliferation. *Exp Cell Res* **169**: 85–94, 1987.
22. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen P and Gabbiani G, Monoclonal antibody against α-smooth muscle actin: New probe for smooth muscle differentiation. *J Cell Biol* **103**: 2787–2796, 1986.
23. Chen K, Heller J and Canellakis ES, Studies on the regulation of ornithine decarboxylase activity by the microtubules: The effect of colchicine and vinblastine. *Biochem Biophys Res Commun* **68**: 401–408, 1976.
24. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
25. Mollard P, Guerineau N, Audin J and Dufy B, Measurement of Ca^{2+} transients using simultaneous dual-emission microspectrofluorimetry and electrophysiology in individual pituitary cells. *Biochem Biophys Res Commun* **164**: 1045–1052, 1989.
26. Darzynkiewicz Z, Sharpless T, Staiano-Coico L and Melamed MR, Subcompartments of the G_1 phase of cell cycle detected by flow cytometry. *Proc Natl Acad Sci USA* **77**: 6696–6699, 1980.
27. Cathala G, Savouret JF, Mendez B, West BL, Karin M, Martial JA and Baxter JD, Laboratory methods. A method for isolation of intact, translationally active ribonucleic acid. *DNA* **2**: 329–333, 1983.
28. Hatch CL and Bonner WM, Direct analysis of RNA in whole cell and cytoplasmic extract by gel electrophoresis. *Anal Biochem* **162**: 283–290, 1987.
29. Maniatis T, Fritsch EF and Sambrook J (Eds.), In: *Molecular loning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, pp. 187–210 and 309–329, 1982.
30. Curran T, Gordon P, Van Beveren C, Teich NM and Verma IM, FBJ murine osteosarcoma virus: Identification and molecular cloning of biologically active proviral DNA. *J Virol* **44**: 674–682, 1982.
31. Kahana C and Nathans D, Nucleotide sequence of murine ornithine decarboxylase mRNA. *Proc Natl Acad Sci USA* **82**: 1673–1677, 1985.
32. Hofbauer R, Müllner E, Seiser C and Wintersberger E, Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the cDNA. *Nucl Acid Res* **15**: 741–752, 1987.
33. Chiou CY and Malagodi MH, Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N*,*N*-Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br J Pharmacol* **53**: 279–285, 1975.
34. Hassid A and Oudinet JP, Relationship between cellular calcium and prostaglandin synthesis in cultured vascular smooth muscle cells. *Prostaglandins* **32**: 457–478, 1986.
35. Kojima I, Shibata H and Ogata E, Action of TMB-8 (8-(*N*,*N*-diethylamino)octyl - 3,4,5 - trimethoxybenzoate) on cytoplasmic free calcium in adrenal glomerulosa cell. *Biochim Biophys Acta* **888**: 25–29, 1986.
36. Simpson AWM, Hallam TJ and Rink TJ, TMB-8 inhibits secretion evoked by phorbol ester at basal cytoplasmic free calcium in quin 2-loaded platelets much more effectively than it inhibits thrombin-induced calcium mobilisation. *FEBS Lett* **176**: 139–143, 1984.
37. Kojima I, Kojima K and Rasmussen H, Mechanism of inhibitory action of TMB-8 [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate] on aldosterone secretion in adrenal glomerulosa cells. *Biochem J* **232**: 87–92, 1985.
38. Berridge MJ and Irvine RF, Inositol phosphates and cell signalling. *Nature* **341**: 197–205, 1985.
39. Pian-Smith MCM, Yada T, Yaney G, Abdel el Mota SMA, Wiedenkeller DE and Sharp GWG, Mobilization of Ca^{2+} from intracellular store of pancreatic B-cells by the calcium store blocker TMB-8. *Endocrinology* **123**: 1984–1991, 1988.
40. Nishimoto I, Hata Y, Ogata E and Kojima I, Insulin-like growth factor II stimulates calcium influx in competent BALB/c 3T3 cells primed with epidermal growth factor. *J Biol Chem* **262**: 12120–12126, 1987.
41. Kojima I, Matsunaga H, Kurokawa K, Ogata E and Nishimoto I, Calcium influx: An intracellular message of the mitogenic action of insulin-like growth factor-I. *J Biol Chem* **263**: 16561–16567, 1988.

42. Nilsson J and Olsson AG, Prostaglandin E_1 inhibits DNA synthesis in arterial smooth muscle cells stimulated with platelet-derived growth factor. *Atherosclerosis* **53**: 77–82, 1984.
43. Loesberg C, Van Wijk R, Zandbergen J, Van Aken WG, Van Mourik JA and De Groot Ph G, Cell cycle-dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E_1. *Exp Cell Res* **160**: 117–125, 1985.
44. Kariya K, Kawahara Y, Araki S, Fukuzaki H and Takai Y, Antiproliferative action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **80**: 143–147, 1989.
45. Reilly CF, Kindy MS, Brown KE, Rosenberg RD and Sonensheim GE, Heparin prevents vascular smooth muscle cell progression through the G_1 phase of the cell cycle. *J Biol Chem* **264**: 6990–6995, 1989.
46. Kariya K, Fukumoto Y, Tsuda T, Kawahara Y, Fukuzaki H, Yamamoto T and Takai Y, Inhibition of DNA synthesis by phorbol esters through protein kinase C in cultured rabbit aortic smooth muscle cells. *FEBS Lett* **217**: 69–73, 1987.
47. Huang CL and Ives HE, Growth inhibition by protein kinase C late in mitogenesis. *Nature* **329**: 849–850, 1987.
48. Hansson GK, Jonasson L, Holm J, Clowes MM and Clowes AM, γ-interferon regulates vascular smooth muscle proliferation and Ia antigen expression *in vivo* and *in vitro*. *Circ Res* **63**: 712–719, 1988.
49. Orekhov AN, Tertov VV, Mazurov AV, Andreeva ER, Repin VS and Smirnov VN, "Regression" of atherosclerosis in cell culture: Effects of stable prostacyclin analogues. *Drug Develop Res* **9**: 189–201, 1986.
50. Jonzon B, Nilsson J, and Fredholm BB, Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J Cell Physiol* **124**: 451–456, 1985.
51. Nakaki T, Nakayama M, Yamamoto S and Kato R, α_1-Adrenergic stimulation and β_2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol* **37**: 30–36, 1989.
52. Garg UC and Aviv H, Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* **83**: 1774–1777, 1990.