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STAUROSPORINE, A PROTEIN KINASE C INHIBITOR INTERFERES WITH PROLIFERATION OF ARTERIAL SMOOTH MUSCLE CELLS

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Staurosporine (10 ng/ml and more), a protein kinase C inhibitor, inhibited the serum-stimulated growth of smooth muscle cells. This inhibitory effect proved to be linked to the inhibition of transition from the G_0 to the S phase of the cell cycle, as measured by 5-bromodeoxyuridine incorporation into the nuclei. As this inhibitory effect of staurosporine was restricted to the first 10 hours of the stimulation, reactions which are essential for the signal transduction may be associated with actions of protein kinase C. The possibility that staurosporine may inhibit cell growth via inhibition of this step warrants further attention.

A major event in development of atherosclerosis is proliferation of intimal smooth muscle cells (SMCs) which migrate from the media, and form the intimal plaque (1). Nishizuka reported that the receptor-mediated hydrolysis of phosphatidylinositol is a common mechanism involved in the transduction of various extracellular signals into the cell (2). Protein kinase C is activated by diacylglycerol derived through phospholipase C-mediated hydrolysis of phosphatidylinositol, as linked to receptors for various extracellular signals, including growth factors (3). If protein kinase C is responsible for an early event induced by extracellular signals (4), then this kinase would act as a positive regulator for transition from the quiescent to the growth state of the cell.

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ABBREVIATIONS: SMC, smooth muscle cell; 5-BrdU, 5-bromodeoxyuridine

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Staurosporine, a microbial alkaloid produced by Streptomyces sp. (5), inhibits protein kinase C from the rat brain (6). In addition, this compound has several biological activities such as inhibition of platelet aggregation (7), and promotion of cell differentiation (8).

We report here that staurosporine inhibits the transition of SMC into the S phase when the cells in a quiescent state are stimulated by serum, the result being inhibition of cell proliferation. Thus, protein kinase C-related reactions are probably involved in the signal transduction of serum stimulation in cultured SMC.

MATERIALS AND METHODS

Cell culture; Smooth muscle cells were prepared enzymatically from rabbit aorta, as described (9). The post-confluent cultures of SMC were usually passaged in Eagle's modified essential medium (MEM) supplemented with 10% of fetal calf serum (FCS), then were maintained after multiple passages. Over 30 passages of SMCs were obtained for this study.

Cell counting; A 12-day-culture of post-confluent SMC was used for seeding. The quiescent culture was replated at 3×10^4 cells in 35 mm dishes containing 2 ml of MEM with 1.5% FCS, and incubated another 4 days. These cells were considered to be in the G_0 phase, as indicated by the inability to synthesize DNA and to increase in cell number under these conditions, at least for 4 days. They were stimulated to grow by changing the medium for that supplemented with 10% FCS, without or with staurosporine. The cells were trypsinized and counted in a Coulter cell counter. Cell viability was checked by trypan blue exclusion. Triplicate cultures were used for each experimental points.

Assay for DNA synthesis; The growth-arrested cells plated on glass coverslips at 5×10^3 cells/cm^2 in 35 mm dishes were stimulated to synthesize DNA by transfer to other dishes containing 2 ml of MEM with 10% FCS and 10 μM 5-bromodeoxyuridine (5-BrdU). Indirect immunofluorescent staining for 5-BrdU incorporated DNA was carried out according to Nakamura et al.(10) with minor modification. At the indicated time after incubation, cells on coverslips were quickly rinsed in phosphate-buffered saline (PBS) and fixed in 70% ethanol for 30 minutes at 4°C. For denaturation of the DNA in situ, the cells were treated with 4N HCl for 30 minutes at room temperature. After rinsing three times in PBS, they were treated with 0.1% Triton X-100 for 10 minutes at room temperature to facilitate permeation of antibodies into the nuclei. The slides were rinsed three times in PBS containing 2 mg/ml of bovine serum albumin (PBS-BSA), then incubated with 10 μg/ml of mouse monoclonal antibodies against 5-BrdU (IMMNOTECH S.A.) for 60 minutes at room temperature. After rinsing in PBS-BSA, the cells were stained with FITC-conjugated antibodies against mouse Ig (Amersham), diluted 1:20 in PBS-BSA for 60 minutes at room temperature. The specimens were photographed by fluorescence microscopy, and the percentage of stained cells was calculated by counting more than one hundred cells.

Drug; Staurosporine was obtained from the myceria of Streptomyces sp. according to the method of Omura et al. (5).

RESULTS AND DISCUSSION

The quiescent culture of SMC, which showed a longitudinally oriented and spindle form, was used. The cell growth became apparent 24 hours after stimulation with 10% FCS and the cells increased 4 fold in cell number after 48 hours (Table I). The cells grew exponentially during which the doubling time was about 20 hours. After 72 hours in culture, the staurosporine (10 ng/ml)-treated culture was reduced in cell number by 73% of control. The cells morphologically changed in the presence of a concentration of 50 ng/ml, and detached from the substratum in the presence of 80 ng/ml or over.

We then examined the effect of staurosporine on serum-induced DNA synthesis in cultures of SMC to determine whether the inhibitory effect of staurosporine (up to 10 ng/ml) is due to inhibition of transition from the G_0 to the S phase. In case of SMC stimulated by 10% FCS, most of the cells began to enter the S phase syncronously during 16-20 hours after stimulation, and about 80% ceased to synthesize DNA within 24 hours (Fig.1). Continuous exposure of the cultures to staurosporine dose-dependently inhibited the serum-induced DNA synthesis. This inhibition was 57% of control at 5 ng/ml and 80% at 10 ng/ml. Some of the cells showed a serum-independent DNA synthesis when the quiescent cultures were exposed to fresh medium without serum. Changes in intracellular pH and concentration of several ions such as Na^+ and Ca^{2+} with medium replacement were presumed to have occured, but the association was not examined

Table I: Effect of staurosporine on growth of quiescent SMC stimulated by serum

	cell number ($\times 10^{-4}$ cell/dish)			
	0hr	24hr	48hr	72hr
control	3.0 ± 0.4	4.3 ± 0.2	11.4 ± 1.0	21.1 ± 2.5
str. 10ng/ml	—	4.6 ± 0.8	5.7 ± 0.2	5.7 ± 0.5
50ng/ml	—	3.8 ± 0.2	4.9 ± 0.4	5.1 ± 0.9

The quiescent cultures were exposed to 10% FCS-MEM in the absence or presence of 10 or 50 ng/ml staurosporine. At the indicated time, triplicate cultures were trypsinized and cell number was determined in a Coulter cell counter.

further. Percentage values of serum-independent DNA synthesis were subtracted from those of serum-dependent one.

Staurosporine (10 ng/ml) was added at different times after stimulation by 10% FCS to investigate the time period when staurosporine is effective (Fig.2). The inhibitory effect of staurosporine could still be observed even in case of addition 10 hours after the stimulation. However, the addition of staurosporine at 12 hours was less effective, and practically no inhibition was observed when staurosporine was added after 14 hours or longer. The inhibitory effect of staurosporine is reversible; most of the cells regained the ability to synthesize DNA by withdrawal of staurosporine from the medium (data not shown). Fig.2 shows that staurosporine is effective during the first 10 hours from the start of the stimulation by 10% FCS and that no inhibition is observed when the cells begin to incorporate 5-BrdU into the nuclei in cultures of SMC. Therefore, it is not likely that staurosporine inhibits DNA synthesis itself.

There are data that protein kinase C plays crucial roles in signal transduction for the control of cell proliferation (2),

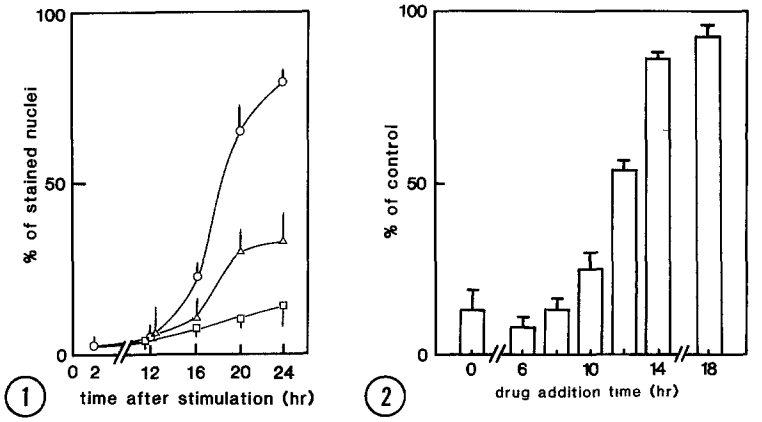

Fig.1 Effect of staurosporine on serum-induced transition from the G_0 to the S phase of SMC in culture. The quiescent cultures were stimulated by transfer to 10% FCS-MEM containing 10 μM of 5-BrdU without(circles) or with 5 ng/ml(triangles), and 10 ng/ml(squares) of staurosporine. At the indicated time, the cultures were fixed in 70% ethanol and processed for immunofluorescent staining analysis of the percentage labeled nuclei (quadruplicate).

Fig.2 Time dependence of the effect of staurosporine on serum-induced transition from the G_0 to the S phase of SMC in culture. Staurosporine (10 ng/ml) was added at the indicated time points after stimulation of the quiescent SMC cultures. After 24 hours of incubation, the cells were fixed in 70% of ethanol and immunostained.

and most activators of this enzyme are mitogenic for many cell types such as Swiss 3T3 (11), lymphocytes (12), thyroid cells (13), chick heart mesenchymal cells (14), and bovine aortic smooth muscle cells (15). It is also suggested that several proteins are phosphorylated in response to exogenous stimulators, such as tumor promotors and growth factors (16 17). This would suggest that activation of protein kinase C occurs in connection with the transduction of extracellular signals. Staurosporine may exert its inhibitory activity against transition from the G_0 to the S phase by inhibiting the protein kinase C activity essential for the signal transduction.

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