

# Phorbol ester stimulation of pancreatic $\beta$ -cell replication, polyamine content and insulin secretion

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Long-term effects of the protein kinase C activating phorbol ester, TPA, on pancreatic  $\beta$ -cell proliferation and insulin production were investigated. It was found that  $\beta$ -cell replication and long-term insulin secretion were enhanced in TPA-treated islets. This was not accompanied by a corresponding increase in (pro)insulin biosynthesis, presumably contributing to the lowered islet insulin content. TPA also increased islet polyamine content but when this increase was prevented by blocking polyamine synthesis, DNA replication and insulin secretion remained elevated. These findings indicate that TPA stimulates  $\beta$ -cell replication and insulin secretion and suggest a stimulatory role for protein kinase C, but not for polyamines, in these processes.

Phorbol ester; Protein kinase C; Pancreatic islet; Insulin secretion; DNA synthesis; Polyamine

## 1. INTRODUCTION

A limited number of substances have emerged as stimulators of pancreatic islet cell proliferation *in vitro* (reviewed by Hellerström and Swenne [1]). Some of these factors, e.g. glucose and polypeptide growth factors, are known in  $\beta$ -cells or other tissues to enhance the activity of protein kinase C (PKC) [2,3]. This phospholipid- and  $\text{Ca}^{2+}$ -dependent enzyme is activated by diacylglycerol which is formed during agonist-induced polyphosphoinositide hydrolysis [4], an event that occurs early in transduction of the mitogenic signal [3]. PKC activation subsequently leads to phosphorylation of the  $\text{Na}^+/\text{H}^+$  antiporter in the plasma membrane which results in intracellular alkalization [5]. PKC activity is subject to stimulation not only by the endogenous compound diacylglycerol, but also by tumor-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [4]. TPA is widely used as an insulin secretagogue in the  $\beta$ -cell [6], but its putative long-term impact on  $\beta$ -cell proliferation and insulin production has not previously been studied. It was the aim of this investigation, by using TPA, to directly study the role of PKC in the regulation of  $\beta$ -cell proliferation and hormone synthesis. The effect of the phorbol ester on the  $\beta$ -cell content of polyamines was also analyzed, since these substances have been shown to be involved in proliferation and macromolecular synthesis by the  $\beta$ -cells [7,8] and numerous other tissues [9].

## 2. MATERIALS AND METHODS

### 2.1. Materials

DFMO was generously given by Dr. Peter P. McCann at Merrell Dow Research Center, Cincinnati, OH, USA. Dr. Juhani Jänne, University of Helsinki, Finland, kindly provided EGBG. TPA was from Sigma and was dissolved in absolute ethanol at a concentration of 10 mM and further diluted in DMSO to stock solutions of 100  $\mu\text{M}$ . All test groups received equal amounts of solvent (0.01%, v/v).

### 2.2. Methods

Pregnant Sprague-Dawley rats belonging to a local stock were killed by cervical dislocation on day 21 of gestation and the fetuses rapidly removed. Fetal rat islets were prepared from pancreatic glands as previously described [10]. At the end of the 5-day culture period groups of 50 islets were transferred to fresh media containing 1% fetal calf serum, with or without 10 nM TPA and polyamine synthesis inhibitors, and cultured free-floating for 3 days.

In this study inhibitors of key enzymes in polyamine biosynthesis were employed, viz. difluoromethylornithine (DFMO) and ethylglyoxal bis(guanylhydrazone) (EGBG). DFMO is a specific and irreversible inhibitor of ornithine decarboxylase, the enzyme regulating putrescine formation [11]. EGBG is a highly selective and potent, albeit not completely specific, inhibitor of *S*-adenosylmethionine decarboxylase, which controls synthesis of spermidine and spermine [12]. Comparisons were made only between control islets and islets treated with DFMO+EGBG. This was done because we have previously shown [8] that ornithine decarboxylase inhibition by DFMO results in partial depletion of putrescine and spermidine, while leaving spermine levels unaffected, and that this decrease does not affect insulin production or  $\beta$ -cell growth.

The methods for analyzing the islet polyamine content [7,8,13], DNA synthesis [8], DNA content [14,15], (pro)insulin biosynthesis and secretion [7,16–18] have been published previously.

## 3. RESULTS

As shown in Table 1 exposure of fetal rat islets for 3 days to 10 nM TPA resulted in a brisk stimulation of DNA synthesis, as measured by [ $^3\text{H}$ ]thymidine in-

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Table I  
Effect of TPA and polyamine synthesis inhibitors on islet DNA synthesis and polyamine content

Islet culture	DNA synthesis (% of control)	Polyamine content (% of control)	
		Spermidine	Spermine
Control	100	100	100
TPA (10 nM)	201 ± 20*	162 ± 18*	138 ± 11*
TPA + DFMO (5 mM)	210 ± 35*	96 ± 14	144 ± 16*
TPA + DFMO + EGBG (100 μM)	185 ± 25*	91 ± 11	103 ± 7

Fetal rat islets were cultured for 3 days in medium RPMI 1640 containing 1% fetal calf serum and supplemented as indicated in the Table. Values are expressed as mean percent of controls ± SEM for 5–6 observations. \*denotes  $P < 0.05$  for chance differences vs. control islets using Student's paired  $t$ -test. Control values were: DNA synthesis,  $1160 \pm 218$  cpm/μg DNA; polyamine content,  $93 \pm 25$  pmol/μg DNA and  $73 \pm 16$  pmol/μg DNA ( $n=6$ ) for spermidine and spermine, respectively.

corporation into DNA. This increase in DNA synthesis was not altered when inhibitors of polyamine biosynthesis (5 mM DFMO + 100 μM EGBG) were added along with the phorbol ester. The islet DNA content was not affected by TPA, DFMO or EGBG (not shown), averaging  $12 \pm 2$  ng/islet.

Following a 3-day exposure to 10 nM of TPA, the islet polyamine content was increased when compared with controls (Table I). Inclusion of DFMO (5 mM) along with the phorbol ester obliterated the increase in spermidine, whereas the elevated spermine content persisted unless EGBG (100 μM) was added as well. When  $\beta$ -cell insulin production was examined after a 3-day exposure to TPA (10 nM), it was found that (pro)insulin biosynthesis was not affected by the phorbol ester (Table II). On the other hand, total protein synthesis was significantly suppressed in TPA-treated islets. Consequently, the fraction (pro)insulin synthesized of total protein was elevated in TPA-treated islets. TPA treatment reduced the islet insulin content (Table I) and further enhanced the accumulation of insulin in the cu-

ture medium. This stimulatory effect of TPA on insulin secretion was not impeded at all by the simultaneous inclusion of DFMO (5 mM) + EGBG (100 μM) (not shown). A short-term glucose challenge at the end of the culture period revealed a prompt insulin secretory response to 16.7 mM glucose in untreated islets, whereas in TPA-treated cells there was a partial attenuation of the glucose-sensitive insulin secretion (Table II). The basal insulin release (in 1.67 mM glucose) was not altered by TPA treatment.

#### 4. DISCUSSION

The calcium- and phospholipid-dependent protein kinase C (PKC) has emerged as a pleiotropic regulator of various cellular functions, including cell proliferation and hormone secretion [4]. This enzyme has been identified as the cellular receptor for tumor-promoting phorbol esters such as TPA [4]. The significance of PKC in regulation of cell proliferation and hormone secretion is amply illustrated by the combined findings of potent

Table II  
Effects of TPA on islet insulin production

	Islet culture	
	Control	TPA (10 nM)
(Pro)insulin biosynthesis ( $10^{-3} \times$ dpm/h per 10 islets)	$8.7 \pm 1.3$	$9.9 \pm 1.2$
Total protein biosynthesis ( $10^{-3} \times$ dpm/h per 10 islets)	$83 \pm 23$	$26 \pm 2.6^*$
(Pro)insulin biosynthesis (% of total protein biosynthesis)	$18 \pm 7$	$39 \pm 5^*$
Insulin content (ng/10 islets)	$339 \pm 75$	$120 \pm 14^*$
Insulin accumulation (% of control)	100	$39 \pm 8^*$
Insulin release in 1.67 mM glucose (ng/h per 10 islets)	$4.0 \pm 0.7$	$3.9 \pm 0.5$
Insulin release in 16.7 mM glucose (ng/h per 10 islets)	$47 \pm 5$	$32 \pm 3^*$

Control cultures accumulated  $88 \pm 26$  ng insulin per 10 islets per ml during the 3-day culture period. Values are means ± SEM for 5–6 observations. \*denotes  $P < 0.05$  for a chance difference vs. controls using Student's paired  $t$ -test.

mitogenic and secretory (and in some cases tumorigenic) actions of TPA and inhibitors of diacylglycerol degradation, and the large increases in PKC activity occurring in response to natural mitogens and secretagogues [2,3]. Further evidence for a crucial role of PKC in normal and neoplastic proliferation is derived from findings of elevated diacylglycerol levels or PKC activity in c-Ha-ras-transformed cells [19,20] and that a mutant PKC obtained from fibrosarcoma cells is able to induce neoplastic transformation of normal fibroblasts [21].

Apart from studies concerned with the significance of PKC in regulation of  $\beta$ -cell activity in the short-term [2,6,22], reports on the long-term influence of PKC stimulation are scarce. However, Schwizer [23] found that exposure of islets isolated from newborn rats to TPA for up to one month enhanced the insulin content of these islets as well as insulin output, without affecting the number of islet cells. In another, more recent study [24] no effects of TPA were found on the DNA synthesis of fetal rat islet cell suspensions, despite stimulation of insulin secretion from these cells. The findings presented here confirm that TPA provokes secretion of insulin from the  $\beta$ -cells, and also suggest that the phorbol ester treatment leads to stimulation of  $\beta$ -cell replication. Because islets prepared according to the presently employed technique contain >90%  $\beta$ -cells it seems reasonable to assume that the results are representative of this particular cell type [10]. In addition, incorporation of [ $^3$ H]thymidine over a 5-h labeling period has been shown to represent  $\beta$ -cell mitotic activity [25]. Because  $\beta$ -cells have a long cell cycle [25], it was considered necessary to expose the islets to phorbol ester for 3 days in order to allow DNA synthesis initiated prior to TPA addition to be terminated before [ $^3$ H]thymidine addition. The fetal islets do not respond mitotically to fibroblast mitogens [8], indicating lack of mesenchymal contamination that might otherwise be expected to obscure  $\beta$ -cell DNA synthesis. There are apparent discrepancies between the present findings and the above-mentioned reports. However, in this study a low concentration of phorbol ester, which was considered specific for PKC activation [4] was used, which could explain the differences in outcome between this study and that of Mourmeaux et al. [24]. In addition, the latter authors utilized suspensions of islet cells isolated directly from fetal pancreas, the purity of which had not been characterized. The discrepancy between the present findings and those of Schwizer [23], who failed to detect any long-term effect of TPA (10 nM) on islet cell number, may be accounted for by an enhanced turnover rate of cells exposed to TPA, which thereby would obscure the mitogenicity of the phorbol ester. Although prolonged exposure to high concentrations of TPA eventually will lead to PKC down-regulation, such changes in enzyme activity are difficult to relate conclusively to changes in  $\beta$ -cell replication and polyamine

content, which occur over 10–24 h. Nonetheless, given the combined facts that PKC overexpression confers an enhanced growth rate (and in some cases neoplastic transformation) in other tissues, and that known  $\beta$ -cell mitogens transiently activate PKC [2,3,19–21], it is likely that the presently observed mitogenicity of TPA indeed reflects PKC activation. In addition, exposure of fetal islets to the highly specific PKC inhibitor, H-7, results in an almost total inhibition of  $\beta$ -cell replication (Åke Sjöholm, unpublished), a finding which is consistent with the proposed role of PKC as a stimulator of  $\beta$ -cell replication.

To my knowledge the present report represents the first attempt to directly investigate the impact of TPA on  $\beta$ -cell insulin production. The findings indicate that the potent secretagogic action of TPA was not compensated for by a corresponding increase in insulin biosynthesis, thus likely contributing to the decreased insulin content in islets exposed to the drug. By the same token, the impairment of glucose-sensitive insulin secretion, that occurred after TPA treatment, likely reflects the lowered insulin content.

In search for possible intracellular mediators of the mitogenic and secretagogic actions of TPA, this study focused on the role of polyamines. This was done because these organic cations have previously been implicated in the regulation of  $\beta$ -cell proliferation and hormone secretion [7,8]. Also, it is well known that increases in polyamine biosynthesis occur whenever cell proliferation is stimulated [9]. The results show the expected increase in islet polyamine content in response to TPA, thereby supporting the concept of a universal accompaniment of cell proliferation by polyamine biosynthesis. However, when attempts were made to elucidate the importance of this increase in polyamines by the use of highly selective inhibitors of key polyamine synthesizing enzymes, it turned out that the mitogenicity and secretory activity of TPA persisted despite prevention of the increases in polyamines in whole islets. This would argue against a significant role for polyamines in conveying the messages of TPA.

In summary, the results presented here indicate that specific PKC activation stimulates not only insulin secretion but also  $\beta$ -cell DNA synthesis, and moreover suggest a lack of participation of polyamines in modulation of these processes. It is suggested that PKC activation may be an important event in transducing the mitogenic signal of glucose and polypeptide growth factors.

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