Modulation of expression of endogenous collagenase and collagen genes by electroporation: possible involvement of Ca^{2+} and protein kinase C

Charles A. LAMBERT, Pascal Y. LEFEBVRE, Betty V. NUSGENS and Charles M. LAPIÈRE*

Laboratory of Experimental Dermatology, University of Liège, Tour de Pathologie – B23/3, CHU Sart Tilman, Belgium

We have investigated the effect of electroporation on the expression of collagen $\alpha 1(\text{I})$, collagenase, *c-fos* and *c-jun* genes in human dermal fibroblasts (HDF), human smooth muscle cells (HSMC) and HeLa cells. Collagenase and collagen mRNA levels were respectively increased and decreased in a voltage-dependent manner in HDF harvested 2 days after a sham electroporation. These effects were still observed 10 days after electroporation. Similar effects occurred in electroporated HSMC. Neither collagen nor collagenase mRNAs were detected in control or electroporated HeLa cells. *c-fos* and *c-jun* mRNA levels were also increased in electroporated HDF, HSMC and HeLa cells har-

vested 1 h after plating. This suggests that factor AP1 (*fos/jun*) could mediate the up-regulation of collagenase expression in electroporated HDF and HSMC. When electroporation of HDF was performed in the presence of H7, an inhibitor of protein kinase C, no increase in collagenase mRNA level was observed, suggesting that protein kinase C might be involved in the transduction of the effect. All the effects reported were also suppressed when cells were electroporated in a medium containing EGTA, suggesting that Ca^{2+} might mediate the transduction of this effect.

INTRODUCTION

The study of the molecular events involved in gene regulation has progressed rapidly since the development of procedures enabling the transfection of promoter/reporter gene constructs into eukaryotic cells and the analysis of the transient expression of the reporter gene. Calcium phosphate/DNA and DEAE-dextran/DNA coprecipitates [1,2], liposomes [3] and electroporation [4] have all been widely used as tools for eukaryotic cell transfection. The latter is considered the most powerful, allowing transfection of cells refractory to other methods.

Very few reports deal with the effects of these techniques on the potential modification of the cell phenotype. Rech et al. showed that electroporation increases DNA synthesis in plant protoplasts [5], and Paulssen et al. [6] demonstrated that production of growth hormone and prolactin was transiently decreased in electroporated pituitary cell lines. In order to investigate the cis-acting elements involved in the regulation of type-I collagen and collagenase genes in response to various stimuli, it was first necessary to know if the basal expression of these endogenous genes could be modified by the procedures used for transfection. We focused our attention on electroporation and showed that it transiently affected expression of collagenase, type-I collagen, *c-fos* and *c-jun* genes. Our data suggest that this effect might be mediated by protein kinase(s) activation, presumably protein kinase C, and increased cytosolic free Ca^{2+} , probably by its release from intracellular stores.

MATERIALS AND METHODS

Chemicals

1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine (H7), EGTA, phorbol 12-myristate 13-acetate (PMA) and AlCl_3 were from Sigma. Dulbecco's modified Eagle's medium (DMEM) was from Gibco and fetal-calf serum (FCS) was from Boehringer.

Cells

Human dermal fibroblasts (HDF) were obtained from a healthy donor aged 18 years and used at passages 7 to 13. Human smooth muscle cells (HSMC) were obtained from umbilical veins and used at passage 7. HDF, HSMC and HeLa cells were cultured routinely in standard culture medium (DMEM supplemented with 10% FCS) at 37 °C and 5% CO_2 . All cells were mycoplasma-free.

Electroporation

Cells were detached with 0.1% trypsin in PBS, centrifuged and collected in culture medium containing FCS except when otherwise indicated. Electroporation was performed without DNA by using a Cellject apparatus (Equibio, Liège, Belgium) at a capacitance of 1500 μF , resistance infinite and the indicated voltages. Cuvettes for electroporation used aluminium-plated electrodes. All solutions were kept at room temperature except when otherwise indicated. After electroporation, the cells were quickly poured into the dishes containing culture medium and transferred to the incubator. In some experiments, cells were pretreated with 50 μM H7 for 45 min before treatment with trypsin and electroporation and collected in culture medium also containing 50 μM H7.

mRNA hybridization

Purification of RNAs, Northern blots, cDNA labelling and filter hybridization have been previously described [7]. Probes were fragments of plasmids Hf677 [$\alpha 1(\text{I})$], ColK4 (collagenase), pRSV-jun (jun) and pcfos (human)-1 (*fos*). Some filters were dehybridized by heating in a solution of $0.05 \times \text{SSC}$ ($20 \times \text{SSC}$ is 3 M NaCl plus 0.3 M sodium citrate) and 1% SDS for several hours at 80 °C and rehybridized using the standard procedure.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; H7, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine; HDF, human dermal fibroblasts; HSMC, human smooth muscle cell; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed.

Figure 1 Increase in expression of endogenous collagenase gene and decrease in expression of collagen $\alpha 1(I)$ genes in HDF and HSMC as a result of electroporation

Cells were electroporated at the indicated voltages, replated and harvested 2 days later. (a), (b) Ratios of the levels of collagenase (a) and collagen (b) mRNA measured in electroporated versus control HDF are plotted against the voltage used. (c), (d) Autoradiograms of Northern blots of RNA extracted from HSMC hybridized with collagenase (c) and collagen $\alpha 1(I)$ (d) probes. Lane 1, control; lane 2, 150 V; lane 3, 300 V.

RESULTS

Effects of electroporation on expression of collagenase and collagen genes

HDF were electroporated at increasing voltages, from 150 V to 300 V by steps of 30 V. Cells handled in parallel but not electroporated were used as controls. Electroporated and control cells were harvested 2 days after replating and RNA was prepared as described. Northern blots (5 μ g/lane) hybridized with a human collagenase probe indicated that collagenase mRNA steady-state level was higher in cells electroporated at each voltage tested than in controls (Figure 1a). This induction steadily increased as a function of the voltage up to 270 V and was sharply augmented at 300 V. The induction of the collagenase mRNA level varied between 4.1- and 17.5-fold (average 11.3 ± 5.7 -fold) in five independent experiments when cells were electroporated at 300 V. A similar experiment was performed at 150 V and 300 V using HSMC (Figure 1c). As was observed for HDF, the collagenase mRNA level increased in HSMC, by a factor of 2 at 150 V and 2.5 at 300 V. In contrast, in HeLa cells, which do not constitutively produce collagenase, no collagenase mRNA could be detected in either non-electroporated or electroporated cells (results not shown). Filters were dehybridized and rehybridized using a collagen $\alpha 1(I)$ cDNA probe. In contrast with collagenase, $\alpha 1(I)$ collagen mRNA level was reduced in a voltage-dependent manner in electroporated HDF (Figure 1b) and HSMC (Figure 1d) compared with control cells (respectively by a mean factor of 2.5 in two independent experiments and 2.9 at 300 V). As expected, no $\alpha 1(I)$ collagen mRNA was detected in HeLa cells (not shown).

Cells electroporated at 300 V were harvested 1, 2, 3, 5 and 10 days after replating. The up-regulation of collagenase mRNA

was 2.3-fold, 4.5-fold, 6.4-fold, 1.6-fold and 1.3-fold respectively (means of two independent experiments; results not shown), indicating that this effect peaked at day 3 or 4.

Effect of electroporation on expression of *c-fos* and *c-jun* genes

The transcriptional factor AP1 (*fos/jun*) is involved in the regulation of collagenase-gene transcription in response to activation of protein kinase C (PKC) [8–11]. HDF, non-electroporated or electroporated at 150 V or 300 V, were harvested 1 h after plating and the level of mRNA coding for *c-fos* and *c-jun* was assayed by Northern blot. Figures 2(a) (*c-jun*) and 2(d) (*c-fos*) illustrate the results of a representative experiment. As compared with the control cells, *c-jun* mRNA was increased by a factor of 2.6 ± 0.7 at 150 V and 11.7 ± 3.2 at 300 V (means $\pm$ S.E.M. of four independent experiments). Under the same conditions, the *c-fos* mRNA was increased by a factor of 3.4 ± 2.5 and 7.1 ± 5.9 respectively. Similar results were obtained with HSMC (Figures 2b and 2e) and HeLa cells (Figures 2c and 2f).

In an attempt to understand the basis of the modifications produced by electroporation, the level of *c-jun* and *c-fos* mRNA was measured in HDF electroporated in either culture medium containing serum or PBS maintained at room temperature or at 4 °C. The cells were harvested 1 h after replating. Similar variations in the expression of these two genes were observed under these different conditions of electroporation (results not shown).

Effect of PKC inhibitor

The expression of collagenase mRNA is increased in response to a wide variety of factors known to increase PKC activity,

Figure 2 Increase in expression of endogenous *c-jun* and *c-fos* genes in HDF, HSMC and HeLa cells as a result of electroporation

Autoradiograms of Northern blots of RNA extracted from control of electroporated HDF (a, d), HSMC (b, e) and HeLa cells (c, f) harvested 1 h after replating. (a)–(c) *c-jun* probe; (d)–(f) *c-fos* probe. Lane 1, control; lane 2, 150 V; lane 3, 300 V.

Table 1 Suppression of the regulation of gene expression when cells are electroporated in the presence of EGTA

HDF were electroporated at 300 V in PBS, Ca^{2+} -free PBS (PBS—) or in Ca^{2+} -free PBS containing 10 mM EGTA (PBS–EGTA), replated and harvested after 1 h (*c-jun* and *c-fos*) or 2 days [collagenase and collagen $\alpha 1(\text{I})$]. mRNA levels were measured by hybridization of Northern blots. Gene expression in naive cells was taken as 100%.

	Gene expression			
	<i>c-jun</i>	<i>c-fos</i>	Collagenase	Col1A1
Control	100	100	100	100
Electroporation in				
PBS	726	930	1434	43
PBS—	695	1067	1566	61
PBS–EGTA	187	300	162	103

including tumour necrosis factor α , epidermal growth factor and the tumour promoter PMA [8,9,12]. To assess the involvement of PKC in the modifications reported above, HDF were electroporated in the presence of 50 μM H7, which is known to inhibit PKC. Naive cells were used as negative controls and cells induced with 100 nM PMA were prepared to assess the inhibitor activity of H7. The level of collagenase mRNA was measured in cells harvested 2 days after replating. Collagenase-gene expression was increased 9.2-fold when cells were treated with PMA and 17.3-fold when cells were electroporated (not shown). Treatment of cells with H7 suppressed this effect by 90% (cells

treated with PMA) or 100% (cells electroporated; not shown), supporting therefore the contention that electroporation induces an increase in PKC activity.

Effects of Ca^{2+} and Al^{3+}

HDF were electroporated in complete or Ca^{2+} -free PBS in either the absence or presence of 10 mM EGTA (Table 1). In the latter case cells were replated after electroporation in culture medium containing 20 mM Ca^{2+} . The levels of *c-jun* and *c-fos* mRNA were measured 1 h after replating (Table 1). The levels of the two proto-oncogenes were increased severalfold and to a similar extent when cells were electroporated in complete or Ca^{2+} -free PBS. In contrast, a sharp decrease in the level of *c-jun* and *c-fos* mRNA was observed when cells were electroporated in Ca^{2+} -free PBS containing EGTA. A similar suppression of the modifications induced by electroporation was observed for the collagenase and collagen $\alpha 1(\text{I})$ mRNA levels measured in cells similarly treated and harvested 2 days after replating.

Cullen and co-workers [13] had previously shown that solubilization of Al^{3+} from the electrode plates during electroporation of lymphoma cells caused an intracytoplasmic increase in Ca^{2+} . No effect of AlCl_3 (from 10 μM to 200 μM) on the levels of mRNA coding for procollagen $\alpha 1(\text{I})$ chain or for collagenase in HDF permeabilized by digitonin as described [13] could be observed in our cells (results not shown).

DISCUSSION

We have demonstrated that the expression of *c-fos*, *c-jun*, collagenase and $\alpha 1(\text{I})$ genes in HDF and HSMC is extensively modified by the electric shock that occurs in electroporation. These modifications could be suppressed or reduced by EGTA suggesting that an increase in the concentration of intracellular Ca^{2+} plays a key role in the effects elicited by electroporation. In addition, since identical results were obtained when cells were electroporated in either complete or Ca^{2+} -free PBS, the entry of extracellular Ca^{2+} into the cells does not seem to be involved in the process, suggesting that Ca^{2+} might be released from intracellular stores. With respect to this, Ozawa et al. [14] have shown that the stimulation of DNA synthesis in mouse osteoblast-like cells by an electric field involved Ca^{2+} ions. Cullen et al. [15] also demonstrated that, in mouse lymphoma cells, electroporation induced the conversion of $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$, producing a release of Ca^{2+} . More recently, the same group demonstrated that this conversion was related to the solubilization of Al^{3+} from the electrode plates [13]. Our results do not support the hypothesis of an involvement of Al^{3+} in the regulation of procollagen $\alpha 1(\text{I})$ chain or collagenase in response to electroporation. Disturbances in the cell membranes by the electric shock might lead to a release of Ca^{2+} from internal stores. Other possibilities such as variations in cytoplasmic pH or electrochemical modifications of cytoplasmic solutes could also be considered. It cannot be excluded that different pathways coexist. Some potential modifications related to the procedure can also be ruled out. Heating is not responsible, since the effect is similar in cells maintained at 37 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$. Diffusion of proteins from the medium is also unlikely as the effect is observed in PBS alone. Our preliminary data indicate that other procedures used for transfection, namely DEAE-dextran, liposomes and chloroquine also increase collagenase-gene expression (C. A. Lambert, unpublished work).

It is known that *c-fos* and *c-jun* oncoproteins activate the transcription of the collagenase gene through association in AP1 and its binding to a specific sequence of the promoter, as

previously shown [8–11]. A consensus AP1-binding site exists in the first intron of the $\alpha 1(I)$ collagen gene [16]. There is, however, no evidence that AP1 can trans-repress the expression of the gene coding for collagen $\alpha 1(I)$. Numerous cytokines have been shown to enhance the expression of *c-fos* and *c-jun* genes by increasing the activity of the Ca^{2+} -dependent PKC. We hypothesize that the increase in *c-fos* and *c-jun* mRNA in response to electroporation occurs via an identical pathway. The inhibition of the induced expression of the collagenase gene when electroporated cells were pretreated with H7 supports this notion. However, since H7 is not completely specific for PKC, we cannot rule out the possibility that other protein kinase(s) play a role in this process. Increased PKC activity has also been shown to decrease $\alpha 1(I)$ collagen gene transcription in human lung fibroblasts [17], also supporting the idea that PKC activity is increased in response to electroporation.

We observed an increased expression of *c-fos* and *c-jun* genes in response to electroporation in cells of various origins, namely HDF, HSMC and HeLa cells. It is conceivable that numerous other cell types share the same responsiveness. The observation of an up-regulation of *c-fos* and *c-jun* genes in electroporated HeLa cells shows that the absence of expression of collagenase and collagen $\alpha 1(I)$ is related to a cell-specific repression of the genes rather than a lack of responsiveness to electroporation. As AP1 regulates the expression of numerous genes, its activation by procedures used for DNA transfection could lead to misinterpretation of the results obtained from transient gene-expression experiments.

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