

# Modulation of cytosolic protein kinase C activity by ferricyanide: priming event seems transmembrane redox signalling

## A study on transformed C3H/10T1/2 cells in culture

A.N. Malviya and P. Anglard

*Centre de Neurochimie du CNRS, Université Louis Pasteur, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France*

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Transformed 3T3/10T1/2 cultured cells incubated with ferricyanide caused a decrease of 2 mM EDTA extractable cytosolic protein kinase C activity in 2 min, whereas 5 or 20 min ferricyanide treatment reverted the enzyme activity to that observed without ferricyanide. The ferricyanide effect in 2 min was abolished by amiloride and sustained by ouabain. Thus, deactivation-activation of cytosolic protein kinase C is attributed to an unknown signal generation during H<sup>+</sup> accumulation coupled with the Na<sup>+</sup>/H<sup>+</sup> exchange phase. In this mechanism the priming event concerns the transmembrane redox process shedding H<sup>+</sup> into the cell interior while impermeant ferricyanide acts as a unique electron acceptor.

*Protein kinase C    Tumor promotion    Transformed cell    Transmembrane signaling    Ferricyanide  
Na<sup>+</sup>/H<sup>+</sup> antiporter*

### 1. INTRODUCTION

During the past 2 years overwhelming evidence has accumulated demonstrating that the regulation of protein kinase C activity constitutes a major pathway of transmembrane signalling [1]. 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA) or its endogenous analogue diacylglycerol are activators of protein kinase C [2]. However, the prevalent current trend to implicate protein kinase C in all the observed phenomena of TPA warrants cautious overview [3]. Moreover, the details of the molecular basis of phorbol ester activation of protein kinase C remain far from clear. Phorbol ester [4] and a variety of stimuli [5–7] have been shown to translocate protein kinase C in subcellular compartments. The changes in the catalytic properties of the enzyme during subcellular delocalization have been reported [8]. Thus, understanding of the molecular mechanism underlying activation-

deactivation of protein kinase C appears to be of key relevance in unravelling the mechanism of tumor promotion as elicited by phorbol ester.

Here, potassium ferricyanide, a recognized impermeant anion [9], has been shown to modulate 2 mM EDTA extractable cytosolic protein kinase C activity in transformed 3T3/10T1/2 cultured cells. The effect of ferricyanide has been scrutinized in the presence of amiloride – an inhibitor of the Na<sup>+</sup>/H<sup>+</sup> exchanger or ouabain which blocks the Na<sup>+</sup>/K<sup>+</sup> pump. It is hypothesized that during the Na<sup>+</sup>/H<sup>+</sup> exchange phase there occurs generation of an unknown signal causing inactivation of cytosolic protein kinase C activity extractable by 2 mM EDTA, the priming event in this process being a transmembrane redox signalling.

### 2. MATERIALS AND METHODS

Amiloride, ouabain, histone type III-S and

phenylmethylsulfonyl fluoride (PMSF) were from Sigma (USA). Methylcholanthrene-transformed C3H/10T1/2 cells were obtained from Institut de Recherches Scientifiques sur le Cancer, Villejuif, France. Basal medium Eagle was from Gibco Europe (Scotland).

### 2.1. Cell cultures

Cells were cultivated in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin, 25 U/ml; streptomycin, 25  $\mu$ g/ml) in 100-mm plastic petri dishes. All cultures were kept in a humidified atmosphere of 5% CO<sub>2</sub>:95% air at 37°C. The cell line was maintained as described [10]. Experiments were carried out on cells 48–72 h postconfluency. The media were changed each alternate day and usually 3 changes in the medium were necessary for attaining confluence.

### 2.2. Potassium ferricyanide treatment

After removal of Eagle's basal medium, cells were gently rinsed twice each with 2 ml Hepes buffered Krebs-Ringer solution containing: NaCl, 125 mM; KCl, 4.8 mM; MgSO<sub>4</sub>, 1.3 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM; glucose, 1 g/l; Hepes, 25 mM, pH 7.4. Potassium ferricyanide was dissolved in the same buffer. Cells were preincubated in 5 ml Hepes-buffered Krebs-Ringer solution for 20 min in an incubator at 37°C. At termination of preincubation, cells were further incubated with 5 ml of the same buffer for the specified time with or without ferricyanide. Ferricyanide treatment was stopped by gentle decantation of the incubating medium from the petri dish and by rinsing the cells, twice each with 2 ml ice-cold solution containing 20 mM Tris-HCl, pH 7.5, plus 2 mM EDTA. Petri dishes were placed on ice before harvesting. When amiloride or ouabain was used in the experiment preincubation of cells was carried out in Hepes-buffered Krebs-Ringer solution supplemented with amiloride or ouabain as warranted.

### 2.3. Extraction of protein kinase C in 20 mM Tris-HCl (pH 7.5) plus 2 mM EDTA

Cells were harvested in a minimum volume of 20 mM Tris-HCl plus 2 mM EDTA medium (usually 200–300  $\mu$ l was employed) and homogenized in a Potter Elvehjem homogenizer

with 15 strokes at 3000 rpm. The homogenate was centrifuged at 100000  $\times$  g for 1 h. The resulting supernatant contained 2 mM EDTA extractable cytosolic protein kinase C and served as the enzyme source for this study. The enzyme activity was assayed without any storage to avoid loss of activity.

### 2.4. Protein kinase C assay

The activity of protein kinase C reported here is the activity of the enzyme extractable from the cell in 2 mM EDTA as reported by Boynton et al. [11]. Details of protein kinase C measurement have been described [12]. However, the concentrations of the various constituents in one assay were: 1.75 mM calcium, 16  $\mu$ g/ml phosphatidylserine, 20  $\mu$ M ATP (3–5  $\times$  10<sup>5</sup> cpm), 1.0 mM EGTA, 20 mM Tris-HCl buffer (pH 7.5), 5.0 mM MgCl<sub>2</sub>, 2 mM PMSF, 20  $\mu$ g histone type III-S. Incubations were carried out at 30°C for 15 min and the activity of the enzyme is the activity observed in the presence of calcium plus phospholipid over the activity in the presence of calcium alone. Protein was determined according to Lowry et al. [13].

## 3. RESULTS

Fig.1 illustrates 2 mM EDTA extractable protein kinase C activity in transformed 3T3/10T1/2 cells as a function of time upon potassium ferricyanide treatment. Protein kinase C activity showed a linear decrease within 1 and 2 min of ferricyanide incubation. The enzyme activity increased at 5 min and remained almost at the same level when cells were incubated for 10 or 20 min with ferricyanide. The value of 2 mM EDTA extractable protein kinase C activity observed from 5 to 20 min of exposure of the cells to ferricyanide was very close to that prior to ferricyanide treatment.

Considering the almost 50% decrease in 2 mM EDTA extractable protein kinase C activity within 2 min of ferricyanide treatment and a further rise to a control value at 20 min under similar conditions, further experiments were designed selecting a period of 2 or 20 min ferricyanide exposure. These data are reported in table 1. The effect of ferricyanide was examined in the presence of 2 mM amiloride, an inhibitor of the Na<sup>+</sup>/H<sup>+</sup> exchange system or 1 mM ouabain, a well-known in-

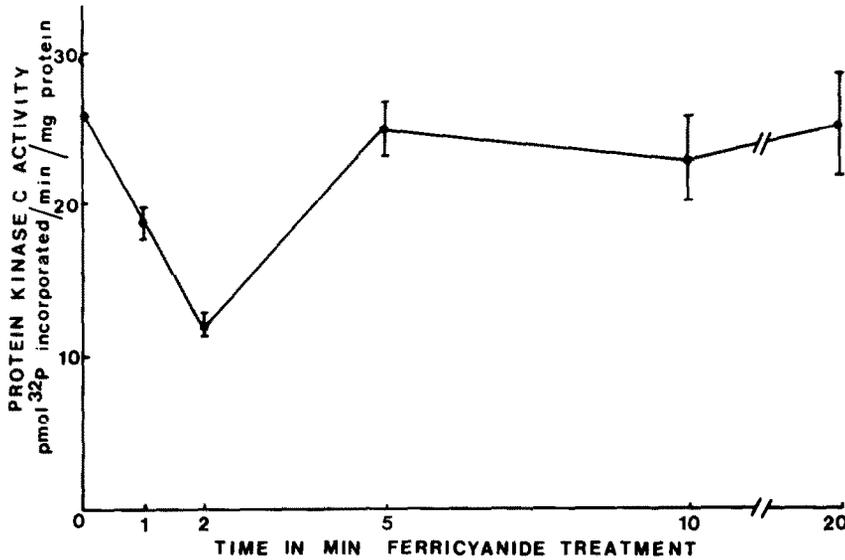


Fig.1. Kinetics of 2 mM EDTA extractable cytosolic protein kinase C activity of transformed C3H/10T1/2 cultured cells incubated with 200  $\mu$ M potassium ferricyanide in Hepes-buffered Krebs-Ringer solution at various times. 48–72 h postconfluent cells were preincubated for 20 min in the same buffer prior to ferricyanide treatment. All incubations were done in an incubator at 37°C. Ferricyanide incubation was terminated by decanting the medium and immediately rinsing the cells attached to the petri dish with an ice-cold solution of 20 mM Tris-HCl (pH 7.5) plus 2 mM EDTA. Cells were harvested in about 200–300  $\mu$ l of the same medium. Cells were homogenized and centrifuged at 100000  $\times$  g for 1 h and protein kinase C activity determined in the resulting supernatant as detailed in section 2. Results are means of 3 individual experiments each done in triplicate.

Table 1

2 mM EDTA extractable cytosolic protein kinase C activity in the presence of amiloride or ouabain with and without ferricyanide treatment of cells in culture

Additions	Protein kinase C activity (pmol/min per mg protein)			
	2 min		20 min	
	– ferricyanide	+ ferricyanide	– ferricyanide	+ ferricyanide
Hepes-buffered Krebs-Ringer solution	24.6 $\pm$ 1.8	11.8 $\pm$ 0.2	25.7 $\pm$ 2.8	25.2 $\pm$ 3.0
Hepes-buffered Krebs-Ringer solution + amiloride	28.8 $\pm$ 4.4	35.8 $\pm$ 4.6	32.7 $\pm$ 5.1	31.5 $\pm$ 5.2
Hepes-buffered Krebs-Ringer solution + ouabain	24.4 $\pm$ 0.7	6.2 $\pm$ 1.6	24.3 $\pm$ 0.9	25.8 $\pm$ 2.2

Cells were preincubated for 20 min with Hepes-buffered Krebs-Ringer solution. In the case of amiloride or ouabain the preincubation medium was supplemented with each one of these. The times of 2 and 20 min are the actual experimental time followed by preincubation irrespective of the presence or absence of ferricyanide (200  $\mu$ M); amiloride (2 mM) or ouabain (1 mM) were added. Other experimental conditions including protein kinase C assay are described in section 2. The data are means of 3 separate experiments performed in triplicate  $\pm$  SD

hibitor of the  $\text{Na}^+/\text{K}^+$  exchange pathway. Cells were incubated with Hepes-buffered Krebs-Ringer solution with amiloride or ouabain, in the presence as well as absence of ferricyanide. It became obvious from these data that the 2 mM EDTA extractable cellular protein kinase C activity was profoundly influenced by ferricyanide. The observed ferricyanide effect was reversed by amiloride, suggesting that inhibition of the  $\text{Na}^+/\text{H}^+$  antiporter abolished the decrease seen in protein kinase C activity within 2 min.

In contrast to the amiloride effect, ouabain seemed to potentiate the ferricyanide effect, i.e. there was a further dip in protein kinase C activity in 2 min when cells were incubated with ouabain plus ferricyanide as compared to that with ferricyanide alone. This strongly suggested that ouabain blocked the  $\text{Na}^+/\text{K}^+$  exchange apparatus which in turn augmented the ferricyanide effect. Interestingly enough, no appreciable change in protein kinase C activity was seen at 20 min of ferricyanide treatment irrespective of amiloride or ouabain supplementation of the incubation medium.

These data strongly favour the notion that the ferricyanide-induced modulation of 2 mM EDTA extractable cytosolic protein kinase C activity operates via a mechanism involving the  $\text{Na}^+/\text{H}^+$  antiporter and the  $\text{Na}^+/\text{K}^+$  exchange systems.

#### 4. DISCUSSION

This study seems to unify 3 essential concepts: (i) the extractability of cytosolic protein kinase C by 2 mM EDTA [11] and thus the avoidance of lengthy chromatographic procedures; (ii) ferricyanide impermeability to the cells and serving as an electron acceptor [14]; (iii)  $\text{Na}^+/\text{H}^+$  activity leads to the intracellular pH shifting towards alkalization and consequent stimulation of the  $\text{Na}^+/\text{K}^+$  pump [15].

As depicted in fig.1, when transformed 3T3/10T1/2 cells were incubated with ferricyanide 2 mM EDTA extractable protein kinase C activity decreased up to 2 min. The enzyme activity returned to the value prior to ferricyanide treatment at 5 min and remained so even after 10 or 20 min exposure of the cells to ferricyanide. Ferricyanide does not permeate the cell and only accepts electrons. This fact is mainly responsible for the in-

teresting phenomenon observed here. Due to the operation of a transmembrane redox system electrons are donated to ferricyanide while protons are shed into the cell interior. This triggers a shift in intracellular pH towards acid values. Once this is set into motion, it is followed by  $\text{Na}^+/\text{H}^+$  antiporter activity. Thus there seems to occur intracellular pH modulation due to  $\text{Na}^+$  influx. The latter process is accompanied by enhanced  $\text{Na}^+/\text{K}^+$  pump activity. Such a scenario is supported by the data of table 1. Treatment of cells with amiloride plus ferricyanide abolished the decrease in protein kinase C activity seen due to 2 min ferricyanide treatment, meaning thereby that amiloride inhibited  $\text{Na}^+/\text{H}^+$  activity which in turn removed the signal responsible for inactivation of protein kinase C.

Ouabain seemed to potentiate further the ferricyanide effect. Ouabain plus ferricyanide decreased by almost 50% the protein kinase C activity observed in the presence of ferricyanide alone. This may be explained by ouabain blocking the  $\text{Na}^+/\text{K}^+$  pump system which in turn sustained  $\text{Na}^+/\text{H}^+$  antiporter activity.

It is of note that in 20 min no ferricyanide effect was seen. This may be attributed to the cellular response to proceed normally.

Fig.2 depicts a hypothetical scheme to explain the phenomenon of protein kinase C activation-deactivation triggered by ferricyanide in 2 min. Although this may sound provocative, it is still postulated that there is a generation of some unknown signal 'X' during  $\text{H}^+$  accumulation coupled with  $\text{Na}^+/\text{H}^+$  exchange causing a decrease in cytosolic protein kinase C activity (fig.2A). Signal X is dissipated by introducing amiloride into the system (fig.2B) and sustained by ouabain (fig.2C). Since ouabain blocks  $\text{Na}^+/\text{K}^+$  pump activity the possibility of more intense  $\text{Na}^+/\text{H}^+$  exchange activity increased which in turn sustains the signal X and hence, a further decline in protein kinase C activity is seen.

Interest in  $\text{Na}^+/\text{H}^+$  antiporter activity with rise in intracellular pH and protein kinase C is of recent origin [16]. Activation of  $\text{Na}^+/\text{H}^+$  by growth factors, in a variety of cell types, suggested its role in proliferation [17]. Nonetheless, the precise inter-relationship between  $\text{Na}^+/\text{H}^+$  activation and that of protein kinase C remains enigmatic. The observation that TPA induced a rise in intracellular pH, in fibroblasts, was sodium dependent and sensitive

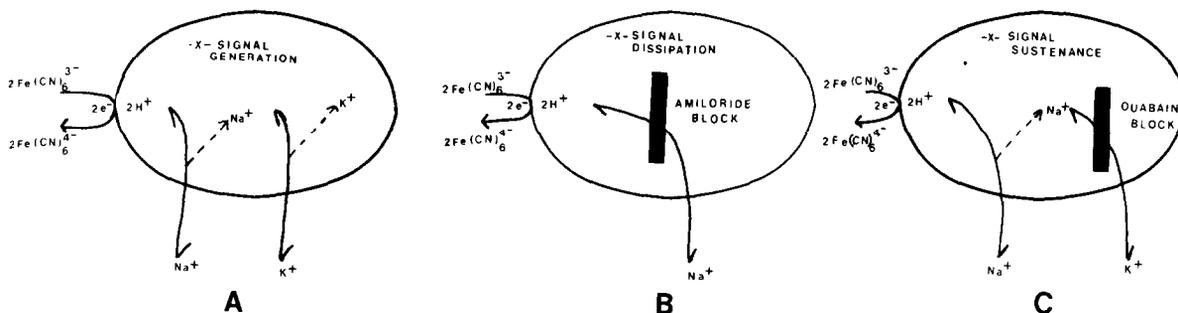


Fig.2. Hypothetical model to explain events associated with activation-inactivation of 2 mM EDTA extractable protein kinase C. (A) Impermeant ferricyanide is recipient of electrons while protons remain in the cell interior. Thus intracellular pH tends towards the acid side. This leads to  $Na^+$  influx due to  $Na^+/H^+$  exchanger activity. Cellular pH shifts towards alkalinization and  $Na^+/K^+$  pump activity is triggered. The observed decrease in cytosolic protein kinase C activity in 2 min due to ferricyanide treatment of cells is attributed to the generation of signal X of an unknown nature during  $H^+$  accumulation coupled with  $Na^+/H^+$  exchange phase. (B) Amiloride block dissipates signal X and thus causes activation of protein kinase C seen in 2 min of ferricyanide plus amiloride treatment of cells. (C) Ouabain blocks  $Na^+/K^+$  pump activity which in turn facilitates  $Na^+/H^+$  exchange system, thereby sustaining signal X and thus protein kinase C activity further decreases when cells were treated with ferricyanide + ouabain as contrasted from ferricyanide treatment in 2 min.

to amiloride analogue [16] indicated that the  $Na^+/H^+$  system may have some bearing on tumor promotion. This leads to the idea that the  $Na^+/H^+$  antiporter is a strong candidate for phosphorylation by protein kinase C, although it has yet to be demonstrated. Notwithstanding, there are indications that in certain cells activation of protein kinase C [18] is not sufficient to activate the  $Na^+/H^+$  antiporter. In the same report it is thought that protein kinase C inhibits some unidentified signal responsible for  $Na^+/H^+$  activation.

Considering the foregoing background the study reported here is of special relevance, since it delineates modulation of cytosolic protein kinase C activity as a function of intracellular pH perturbations linked to certain cellular functions. In our opinion, the pH perturbation triggered by ferricyanide is not without cognizance of cellular metabolic preference. In this context, a recent report [19] showing pH regulation in neutrophils deserves mention.

It will be of great importance to determine whether activation-deactivation of cytosolic protein kinase C by ferricyanide is specific for the cell type studied or is a more general phenomenon entailing other cell types. In such an eventuality this study advances a simple, yet useful model for ad-

ressing the question of the molecular regulation of protein kinase C activity which happened to assume a key pathway of transmembrane signaling coupled to tumor promotion.

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