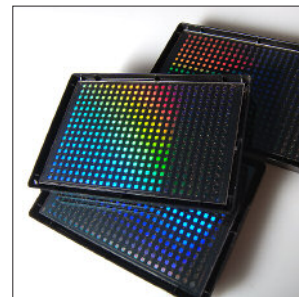


Corning® Epic® Cell-Based Assays: Development of a Label-Free Cell-Based Assay for Protein Kinase C Inhibition



SnAPPShots

A brief technical report
from the Corning
Applications Group

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Introduction

Corning Epic technology is a label-free platform based on optical biosensor technology for investigating biological processes *in vitro*. It enables high-throughput, label-free detection in both cell-based and biochemical assay formats. Epic technology consists of an SBS-standard 384 well microplate with integrated optical biosensors, and a high-throughput screening-compatible microplate reader that can be operated as a standalone device for assay development, or integrated with other HTS equipment.

Cell signaling is an integrated process, and crosstalk between pathways is a common phenomenon. Epic technology provides insight into the integrated effects of a small-molecule or peptide modulator, as the quantitative downstream signal results from cell morphology changes and dynamic mass redistribution (DMR) of proteins within the cell (1,2). The ability of Epic technology to detect endogenous receptor signaling means that assays can be carried out with genetically unmodified cells. This obviates the need to generate stable cell lines overexpressing the receptor of interest.

Protein kinase C (PKC) has an important physiological role in several indications ranging from diabetic neuropathy to transplant rejection, inflammation, neurodegeneration and cancer. Traditionally, the activity of PKC has been evaluated in biochemical enzymatic assays using purified enzyme and peptide substrates, or in cell-based assays monitoring only a

very specific and limited downstream event (calcium flux (3), cellular imaging (4) or adrenocorticotrophin (ACTH) secretion (5) for example). In this application note, the development of a new label-free cell-based assay for endogenous PKC activity is described. It is shown that intracellular PKC activity is inhibited using specific PKC inhibitors, and demonstrated that Epic technology is well suited for the development of a cell-based assay for endogenous PKC activity. Therefore, it is proposed as a new method to screen for kinase inhibitors using a more physiologically relevant set-up with great potential for drug discovery.

Materials and Methods

Cell Culture

HEK-293 cells were cultured in DMEM containing 10% FBS and antibiotic/antimycotic in 5% CO₂ at 37°C. The cells were plated at 15,000 cells/well in 40 µL of media, and incubated overnight at 37°C on Corning Epic 384 Well Fibronectin-Coated Cell Assay Microplates (Cat. No. 5042).

Assay Details

The cells were washed and equilibrated with 20 µL of assay buffer (HBSS, 20 mM HEPES, 0.2% DMSO) for 2 hours. Following equilibration of the microplates on the Epic carousel for 1 hour, a baseline measurement was recorded. The cells were then treated with varying concentrations of phorbol myristate acetate (PMA), a known activator of PKC. Kinetic measurements were taken for 60 minutes after PMA addition, and the EC₅₀ for PMA was calculated from the DMR change at 15 minutes post-addition. To evaluate the effects of different PKC inhibitors, the cells were equilibrated in assay buffer (HBSS, 20 mM HEPES, 0.2% DMSO) for 1 hour inside the Epic carousel at 26°C before treatment with the inhibitors. The inhibitors were incubated with the cells for 2 hours prior to addition of EC₈₀ concentration of PMA. All of the inhibitors are described as cell-permeable inhibitors of PKC. The IC₅₀ of PKC inhibition was calculated from the DMR change at 15 minutes post-addition of PMA.

Reagents

PKC inhibitors were obtained from Sigma-Aldrich® and Calbiochem.® HBSS, HEPES, cell culture media, and reagents were obtained from Invitrogen. PMA was obtained from Sigma-Aldrich. Data analyses were carried out using GraphPad Prism® (Version 5) software.

Product	Vendor	Cat. No.
DMEM	Invitrogen™	12800-017
FBS	Invitrogen	10270-106
Antibiotic/antimycotic (Penicillin/Streptomycin)	Invitrogen	15140-122
Epic® 384 Well Fibronectin-Coated Cell Assay Microplates	Corning	5042
HBSS	Invitrogen	14025-126
HEPES	Invitrogen	15630-080
DMSO	Sigma	D2650
PMA	Sigma	P8139
Bisindolylmaleimide II	Sigma	B3056
Ro-31-6233 (Bisindolylmaleimide IV)	Sigma	B3306
Ro-31-7549 acetate (Bisindolylmaleimide VIII)	Sigma	B3806
Ro-31-8425 (Bisindolylmaleimide X)	Sigma	B3931
GF109203X hydrochloride	Sigma	B6292
Gö 6983	Sigma	G1918
Ro-32-0432	Calbiochem	557525

Results

Determination of EC₅₀ of PMA

HEK-293 cells were treated with varying concentrations of PMA, a known PKC activator. The Epic response (positive DMR) upon PMA addition was monitored. The EC₅₀ depends on the time point selected to evaluate the change in DMR. The profile of the response is different in cells treated with lower concentrations of PMA as compared to the high doses. The change in DMR with high dose of PMA saturates within the first 15 minutes while the DMR change with the lower dose of PMA increases up to 60 minutes. This explains the difference in EC₅₀ calculated at different time points (data not shown). However, since a dose-dependent inhibition with the PKC inhibitors is observed at 15 minutes, 15 minutes post-compound addition has been selected as the time point for EC₅₀ calculation.

The EC₅₀ of the PMA dose response was found to be 0.27 μM (Fig. 1), up to 10-fold higher than the reported EC₅₀ (6-9). These differences may result from the fact that Epic technology measures the integrated cell response whereas the other methods rely on single event measurements.

Determination of the effects of different PKC inhibitors

To study dose responses with different PKC inhibitors, HEK-293 cells were treated with varying concentrations of different small-molecule PKC inhibitors for 2 hours,

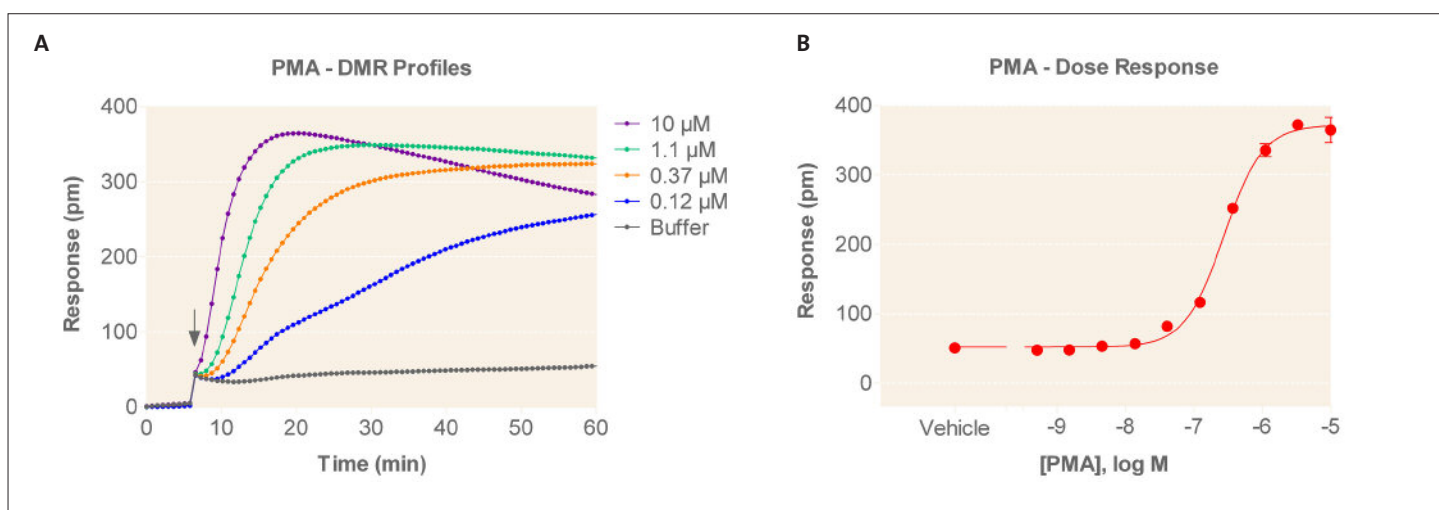


Figure 1. Determination of EC₅₀ of PMA. (A) The Epic response (pm) due to DMR in response to PMA addition to HEK-293 cells is shown. The arrow indicates the point of addition of PMA to the cells. Traces are from one representative experiment. (B) The EC₅₀ of the PMA dose response was calculated from the Epic response (pm) value at 15 minutes post-PMA addition, and found to be 0.27 μM. Error bars represent the mean and SEM of triplicate data points.

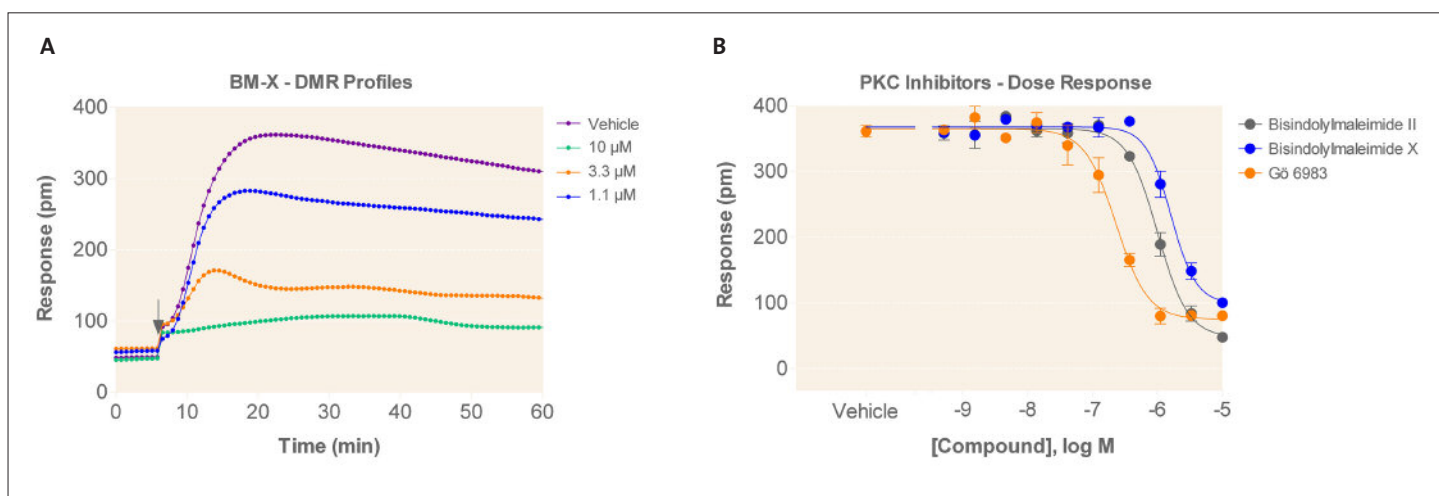


Figure 2. Inhibition of PKC with small-molecule inhibitors. (A) DMR changes in the PMA profile in response to varying concentrations of the PKC inhibitor Bisindolylmaleimide X are shown. The arrow indicates the point of addition of PMA to the cells. Traces are from one representative experiment. (B) Representative inhibition profiles of a few PKC inhibitors are shown. The IC_{50} of inhibition of the PMA dose response was calculated from the Epic response (pm) value at 15 minutes post-PMA addition. Error bars represent the mean and SEM of triplicate data points.

Table 1. IC_{50} values obtained with different PKC inhibitors: Comparison with literature values

PKC Inhibitor	IC_{50} (nm) for PKC isoforms α , β_I , β_{II} and δ (data from biochemical assays)	Mean IC_{50} (nm) on Epic
Bisindolylmaleimide II	13; isoform selectivity unavailable (6)	800
Bisindolylmaleimide IV (Ro-31-6233)	87-5000; isoform selectivity unavailable (7)	1800
Bisindolylmaleimide VIII (Ro-31-7549)	53, 195, 163, 175 (8)	580
Bisindolylmaleimide X (Ro-31-8425)	8, 8, 14, 39 (7,8)	1200
GF 109203X HCl	20, 17, 16, NR (6)	1770
Gö 6983	7, NR, NR, NR (9)	136
Ro-32-0432	9, 28, 31, 108 (8)	2900

NR = Not reported.

followed by addition of PMA to an EC_{80} concentration (~2 μ M). The IC_{50} of inhibition of the PMA response was calculated from the Epic® response at 15 minutes post-PMA addition (Fig. 2 and Table 1). No DMR response was observed when the cells were treated with any of the inhibitors alone and background response of the inhibitors was at equilibrium after 2 hour incubation (data not shown).

As seen in Table 1, the IC_{50} values obtained using Epic technology are mostly in the micromolar range; in the literature, few reported IC_{50} values are available for PKC inhibitors, and

most of the data available are for biochemical assays (binding or enzymatic assays). The data obtained here, therefore, vary substantially from reported data, as the IC_{50} values measured using Epic technology are representative of intracellular events very different from direct phosphorylation of peptide substrates in solution-based biochemical assays (6-9), or from single-event readouts in orthogonal cell-based assays. Furthermore, HEK-293 cells are described to contain highly expressed PKC α , β_I , β_{II} (10), and these levels might be different in other cell types and different culture conditions leading to variations in the inhibition constant observed.

Epic® technology provides several advantages over common orthogonal assay formats for kinase activity. Whereas direct phosphorylation assays provide protein-ligand affinity and inhibition constants, the Epic label-free cell-based method described here measures a full cellular response, leading to potentially higher-content information for drug discovery (11).

Conclusions

- ▶ Epic technology is well suited for the development of a cell-based assay for endogenous PKC activity: PKC activity is inhibited using specific inhibitors in a label-free cell-based assay format.
- ▶ Epic technology offers a new and robust screening method for kinase inhibitors in high-throughput cell-based assays that could be applied in various therapeutic areas from cancer to HIV research.
- ▶ The proposed assay format could potentially provide more physiologically relevant data for the identification of new drug candidates.
- ▶ The high detection sensitivity of Epic technology enables pharmacological characterization of endogenously expressed targets in live cells.

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