FlashPlate® File #6

Use of FlashPlate for Automated Kinase Assays

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Abstract

A novel assay procedure for measuring Ser/Thr and Tyr protein kinase activity has been developed which is applicable for automated high throughput screening. The new method is based on the FlashPlate platform, a 96-well polystyrene microplate with plastic scintillant coated wells, from PerkinElmer Life Sciences. The method combines special features of FlashPlate with a proprietary assay protocol. The usefulness of the assay procedure was evaluated by measuring the activity of protein kinase C (PKC) (Ser/Thr protein kinase) and a vascular endothelial growth factor (VEGF) receptor containing a tyrosine kinase insert domain (KDR). It is anticipated that the assay protocol can be easily adapted to other Ser/Thr and protein kinase assays.

Introduction

Protein kinases are key regulators of a great variety of physiological and pathophysiological processes, including tumor development. There is consequently great interest in identifying specific inhibitors of particular protein kinases which can be developed as novel antitumor agents.

One kinase involved in tumor growth is PKC, a family of 11 closely related Ser/Thr kinases[1]. It is not entirely clear whether all subtypes of PKC contribute to tumor development, or whether one subtype plays a particular role as a key regulator of tumor promotion.

Another protein kinase shown to contribute to tumor development is the cytoplasmic domain of KDR. This receptor is a member of the great family of growth factor receptors with intrinsic tyrosine kinase activity. Tumor cells can release VEGF, which subsequently acts as a specific mitogen for vascular endothelial cells. VEGF-induced proliferation and migration of endothelial cells results in the formation of novel blood vessels, a prerequisite for growth of unvascularized tumor cell clones[2].

In order to identify specific inhibitors of PKC isozymes and KDR Tyr protein kinase, an assay was developed to allow automated FlashPlate-based high throughput screening of compound libraries (up to 100,000 substances or extracts).

Methods

To discuss details of the assay procedure and the possibility of custom testing, please contact Dr. C. Schächtele (phone +49-761-206-1710; fax +49-761-206-1705).

The PKC reaction cocktail contained the enzyme and:

    50 mM HEPES-NaOH (pH 7.5)
    5 mM $MgCl_2$
    1 mM EDTA
    1.25 mM EGTA
    1.32 mM $CaCl_2$
    5 µg/ml phosphatidylserine
    1 µg/ml 1.2-diolein
    1 mM DDT
    1 µM [^{33}P]-γ-ATP (1x10^6 cpm)

The KDR assay mixture contained the enzyme and:

    50 mM HEPES-NaOH (pH 7.5)
    3 mM $MgCl_2$
    3 mM $MnCl_2$
    3 µM Na-orthovanadate
    2 mM DDT
    1 µM [^{33}P]-γ-ATP (1x10^6 cpm)

The reaction mixtures were added to FlashPlate (SMP200) and incubated at 30°C for 1 hour (standard assay) or as indicated in Figure 1. Incorporation of $^{33}P_i$ was determined with a microplate scintillation counter.

Results and Discussions

The applicability of the novel method was analyzed in an initial series of experiments by studying the linearity of the reaction over a period of 90 minutes, for both protein kinases. Figure 1 shows that in both cases the reaction was linear for the entire test. A comparison of basal (o) with total (●) kinase activity demonstrates that almost all measured radioactivity was attributable to specific enzymatic reaction, and not to nonspecific binding of [^{33}P]-γ ATP to FlashPlate (97% for PKC; 99% for KDR). In addition, Figure 1 shows that the amount of specifically incorporated radioactivity obtained after one hour (i.e., 7,800 cpm in case of PKC; 8,300 cpm in case of KDR) is high enough to make this procedure valuable for inhibitor screening.

This potential application was proven by measuring the inhibitory profile of Gö 6850, a well characterized PKC inhibitor[3], in the FlashPlate-based PKC assay. Figure 2 presents a comparison of two inhibitory curves of Gö 6850: one performed with the new FlashPlate-based method, the other performed with a conventional PKC kinase filtration assay. The curves are nearly identical, a fact which clearly substantiates the quality of the new method.

In a further approach, reproducibility of the novel test procedure was studied by measuring ten inhibitor curves with Gö 6850 under identical conditions. For each inhibitor concentration tested, the standard deviation of the mean of inhibition was below 10%. Moreover, a mean IC_{50} value of 11 nM, with a standard deviation of 4 nM, demonstrates the excellent reproducibility of the assay procedure.

In summary, a novel proprietary procedure for measuring kinase activity and screening for kinase inhibitors has been successfully developed on FlashPlate. This new assay can be easily performed with automatic pipetting systems, providing the basis for robotic High Throughput Screening. Moreover, this method potentially can be adapted to assay other Ser/Thr kinases or Tyr protein kinases.

Figure 1a

**Time course of PKC activity in the presence (●) or absence (o) of phosphatidylserine and diolein.**
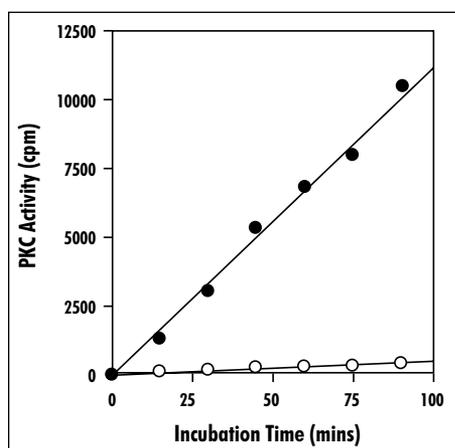
Figure 1b

Time course of KDR tyrosine kinase activity in the presence of active (●) or denatured (o) kinase.
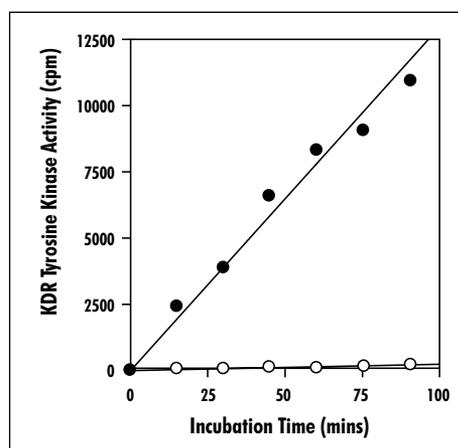
Figure 2

Inhibition of recombinant PKC-γ by Gö 6850. Enzyme activity was calculated as a percentage of control in the absence of inhibitor. One curve (o) was obtained with a conventional procedure, including a separation step by filtration. The other curve (●) was calculated by meaning the results from ten independent experiments performed with the FlashPlate-based method. The calculated IC_{50} values were 15 nM (o) and 11 nM (●), respectively. The bars represent the standard deviation of the mean of inhibition (n=10).
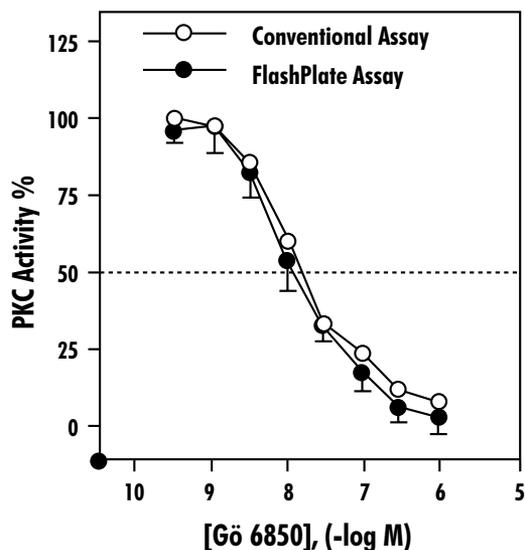
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