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Khellin, but not 8-methoxypsoralen, inhibits adenylyl cyclase system in HeLa cells

Anna Di Stefano [a], Lorenza Trabalzini [a], Rita La Gaetana [a], Luca Parente [b,1], Paola Lusini [a], Paola Martelli [a,*]

[a] *Department of Molecular Biology, University of Siena, Piano dei Mantellini, 44, 53100 Siena, Italy*
[b] *Department of Pharmacology, IRIS, Siena, Italy*

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Abstract

Until recently, the therapeutic effects of furocoumarins and furochromones plus UV-A light were thought to be due to their ability to form photoadducts with DNA in the cell nuclei; now it appears that membrane effector systems may be involved as targets. Here we show that in HeLa cells khellin at 1 and 5 μM final concentration, in combination with UV-A light, inhibits NaF-stimulated adenylyl cyclase activity and Pertussis Toxin (PT)-catalyzed ADP-ribosylation of α-subunits of inhibitory guanine nucleotide regulatory proteins (Gi) and increases GTPase activity. In the same experimental conditions, 8-methoxypsoralen (8-MOP), either alone or plus UV-A, does not affect adenylyl cyclase and GTPase activities. Our results suggest that in HeLa cells, through an interaction with a receptor and the mediation of Gi proteins, the adenylyl cyclase system is a target for khellin but not for 8-MOP.

Keywords: HeLa cell; G-Protein; 8-Methoxypsoralen; Khellin; Adenylyl cyclase

1. Introduction

Since the 1970s, PUVA (Psoralen plus UV-A) therapy has been used for a number of skin diseases (e.g., psoriasis and vitiligo) [1]. More recently, psoralens (also called furocoumarins) have been employed as photochemoprotective agents against the risk of UV-B exposure [2] and in photochemical decontamination of blood products [3]. Finally psoralens, used in 'photopheresis' — an extracorporeal photochemotherapy — act as immunological modifiers against disorders of circulating T-cells [4].

The therapeutic action of psoralens is widely ascribed to the formation of DNA-psoralen photoadducts [5]. However, the effects of the psoralen-membrane phospholipid reaction [6] and the modulation of PLC, adenylyl cyclase [7] and the immune system [8] following PUVA treatment strongly indicate the interactions of psoralens with cell membranes and suggest that these structures represent an important target involved in their clinical effectiveness. All these experimental observations are further supported by the discovery of a membrane receptor for 8-MOP [9] and by the demonstrated biological activity of psoralen analogues deprived of their ability to form the traditional DNA cross-linked double photoadducts [10] Moreover, there is evidence that 8-MOP, either alone or in combination with UV-A light, modulates gene expression [11], and that UV light triggers a number of signalling events at the plasma membrane in mammalian cells (including those that ultimately lead to an increase in gene expression) by modulating transcription factor activity [12–14].

For a better understanding of the molecular mechanism of action, we have studied, in HeLa cells, the behaviour of adenylyl cyclase, PT-catalyzed ADP-ribosylation and the GTPase activity of GTP binding proteins related to the action of adenylyl cyclase, under the effects of two different photochemotherapeutic agents: 8-MOP (commonly used in PUVA therapy), which is a linear psoralen very active in photobinding to DNA and cell membrane proteins and phospholipids [6]; and khellin, a natural furochromone structurally related to psoralens but a poor producer of photoadducts to DNA [15–17].

* Corresponding author. Fax: +39 577 298084.
[1] Present address: Institute of Pharmacology and Pharmacognosy, University of Palermo, Italy.

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2. Materials and methods

2.1. Cell treatment

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin G and 100 mg/ml streptomycin, at 37°C in a controlled humidified incubator in 5% CO_2. $4 \cdot 10^6$ cells were placed on 9 cm plastic Petri dishes and after 72 h the monolayers of HeLa cells were washed twice with serum-free DMEM and incubated again at 37°C for 30 min in the same medium without or with 8-MOP or khellin (1 and 5 μM, final concentrations). Stock solutions of the two drugs were prepared by dissolving them in absolute ethanol ($5 \cdot 10^{-2}$ M) and dilutions with serum-free DMEM were made prior to their addition to the cells. After 30 min, a series of cell culture dishes was kept in the dark, while the others, with the top removed, were irradiated with UV-A light (320–400 nm) with a peak emission at 366 nm. The UV-A source consisted of four black-light tubes (purchased from Helios Italquarz srl; Milano) placed 20 cm above the culture dishes. The incident light was measured with a Centro UVMeßgerät OSRAM International Light UV-radiometer. In all experiments cells were exposed to 2 J/cm^2. The intensity of the UV-A light used corresponds to that employed for therapeutic purposes (and which provides a satisfactory effect). Indeed, unpublished results have shown that lower intensities (0.5–1.5 J/cm^2) lead to a lesser effect, while higher intensities (> 2 J/cm^2) elicit phototoxic side effects.

During the time of irradiation (10 min), the cells were covered with phosphate-buffered saline (PBS). Controls are the cells without drugs and without irradiation. After the exposure, the cells were used for the preparation of membranes.

The viability of the cells, as assayed by Trypan blue exclusion (95% under control conditions), was unaffected by both drugs studied and by UV-A irradiation.

2.2. Membrane preparation

Cells were harvested by scraping in ice-cold PBS and then pelleted at 3000 rpm for 15 min at 4°C. The cells were placed for 5 min in hypotonic buffer consisting of 50 mM Tris-HCl (pH 7.6) and homogenized using a Dounce glass-glass (35 strokes). After centrifugation at 23 000 × g for 20 min at 4°C, the pellet was resuspended in 50 mM Tris-HCl (pH 7.6) to yield a membrane fraction containing 2 mg of proteins per ml as determined by the method of Bradford [18]. The fraction was used promptly (to evaluate adenylyl cyclase activity) or after storage at −70°C (to evaluate ADP-ribosylation and GTPase activity).

2.3. Adenylyl cyclase assay

Adenylyl cyclase activity in basal and NaF (10 mM)-stimulated conditions was measured with the commercially available radio-immunoassay kit (Cyclic [^{3}H]AMP assay system) of Amersham.

2.4. ADP-ribosylation

ADP-ribosylation of HeLa membranes was induced by PT. Prior to its addition to membrane preparations, PT (1 mg/ml) was activated by incubation in 20 mM dithiothreitol (DTT) at 30°C for 30 min. The HeLa membranes (50 μg of proteins) and ROS (rod outer segments) membranes (20 μg) used as an internal control, were incubated at 37°C for 1 h with and without PT (5 μg/ml) in 150 μl

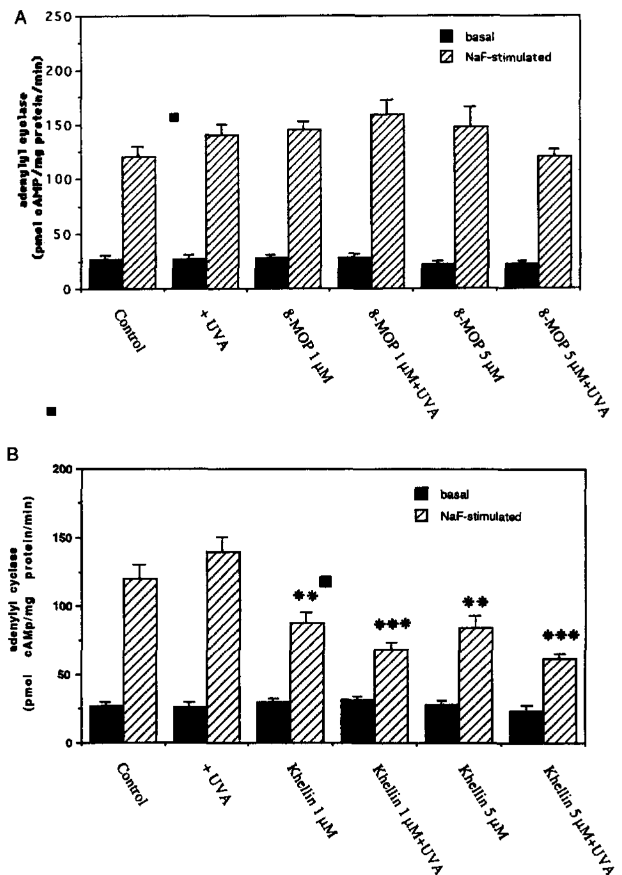

Fig. 1. (A) Effect of 8-MOP, UV-A light and a combination of the two on adenylyl cyclase activity. Cell cultures were treated with the indicated concentrations of 8-MOP with or without UV-A light (2 J/cm^2). Membranes were prepared from control and treated cultures and assayed for adenylyl cyclase activity as described in Section 2. The specific activities of basal and NaF-stimulated adenylyl cyclase are reported. Values represent the mean ± S.E. of 12 measurements (4 assays in 3 separate experiments). NS: $p > 0.05$, compared to control. (B) Effect of khellin, UV-A light and a combination of the two on adenylyl cyclase activity. Cell cultures were treated with the indicated concentrations of khellin with or without UV-A light (2 J/cm^2). Membranes were prepared from control and treated cultures and assayed for adenylyl cyclase activity as described in Section 2. The specific activities of basal and NaF-stimulated adenylyl cyclase are reported. Values represent the mean ± S.E. of 12 measurements (4 assays in 3 separate experiments). ** $p < 0.01$ and *** $p < 0.001$, compared to control.

of a reaction mixture containing: 70 mM Tris-HCl (pH 7.4), 0.7 mM ATP, 0.07 mM GTP, 17 mM thymidine, 1.12 μM [^{32}P]NAD. The ADP-ribosylation was started by the addition of [^{32}P]NAD. The samples (in duplicate) were incubated at 30°C for 60 min and the reaction was terminated by centrifugation for 3 min at 10000 rpm. The radiolabelled membranes were washed with 200 μl of 50 mM Tris-HCl (pH 7.5) and then centrifuged at 10000 rpm. The pellet was dissolved in 30 μl of Laemmli sample buffer and heated for 3 min in a boiling water bath.

The labelled proteins were separated using 12% SDS-polyacrylamide gels [19]. After electrophoresis the gels were placed in a solution of 5% glycerol, 10% methanol and 5% acetic acid for 2 h and then enveloped. The bands labelled with ^{32}P were visualized and quantified by PhosphorImager 400 A (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. GTPase assay

GTPase assay was performed by measuring the release of ^{32}P from [γ-^{32}P]GTP essentially as described by Cassel [20]. The HeLa cell membranes (10–20 μg) were incubated at 37°C for 10 min in 100 μl of reaction mixture containing 50 mM imidazole HCl buffer (pH 6.7), 0.25 μM [^{32}P]GTP (30 Ci/mmol), 5 mM $MgCl_2$, 0.2 mM 5′-adenylyl imidodiphosphate (App(NH)p), 0.1 mM ATP, 0.1 mM EGTA, 2 mM mercaptoethanol, and ATP-generating system consisting of 3 units of creatine phosphokinase and 2 mM creatine phosphate. The assay was begun by addition of [γ-^{32}P]GTP to the mixture which was then incubated for 30 min at 37°C. The reaction was stopped by addition of 0.5 ml of a 5% suspension of Norit A in 20 mM phosphate buffer (pH 7). The mixture was centrifuged at 12000 rpm for 5 min at 4°C and the ^{32}P in the clear supernatant (200 μl) was measured.

The blank value, due to contamination of the substrate by ^{32}P and non-enzymatic hydrolysis of ^{32}P during the assay, was less than 1% of the total radioactivity in the reaction mixture and it was subtracted from all data.

2.6. Statistical analysis

Results are expressed as mean ± S.E. Data were analyzed using Student's t-test; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of adenylyl cyclase in membranes from HeLa cells treated with khellin in the dark and plus UV-A light

Fig. 1 shows that UV-A light alone does not modify adenylyl cyclase. Similarly 8-MOP, at 1 or 5 μM final concentration, fails to affect either basal or stimulated adenylyl cyclase activity in any of the experimental conditions (Fig. 1A). Although cell treatment with khellin (alone or with UV-A) does not significantly alter basal adenylyl cyclase, the combination of khellin and UV-A markedly decreases the NaF-stimulated enzymatic activity; at the higher concentration (5 μM), the inhibitory effect is more evident (Fig. 1B). Stimulated adenylyl cyclase activity is also decreased, to a lesser degree, by khellin 1 and 5 μM alone.

3.2. Cell treatment with khellin or 8-MOP, alone and plus UV-A light, inhibits the subsequent PT-dependent ADP-ribosylation of membrane Giα

In order to investigate whether the inhibition of adenylyl cyclase by khellin is through Gi proteins [21], we evaluated the PT-dependent ADP-ribosylation of mem-

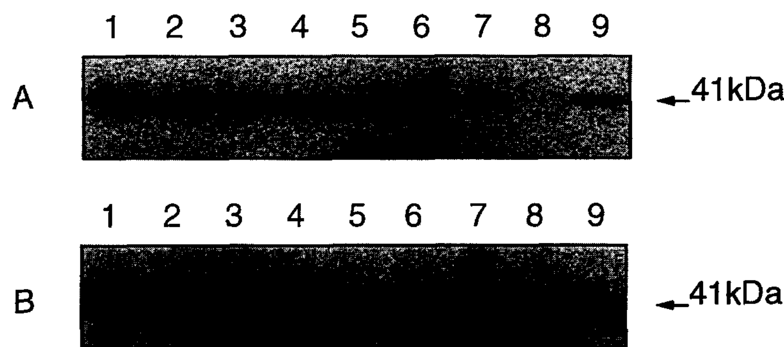

Fig. 2. SDS-polyacylamide gel electrophoresis of HeLa cell membranes after [^{32}P]ADP-ribosylation by PT. Membranes (50 μg) obtained from HeLa cells previously treated as reported in Section 2, were incubated at 37°C for 1 h with PT in 150 μl of a reaction mixture containing 1.12 μM [^{32}P]NAD. The radiolabeled proteins were analysed by PhosphorImager 400. Panel A: control (lane 1), UV-A light (lanes 2 and 3), 1 μM 8-MOP (lane 4), 1 μM 8-MOP plus UV-A light (lane 5), 5 μM 8-MOP (lane 6), 5 μM 8-MOP plus UV-A light (lane 7), without PT (lane 8),ROS membranes (lane 9). Panel B: control (lanes 1 and 2), UV-A light (lane 3), 1 μM khellin (lane 4), 1 μM khellin plus UV-A light (lane 5), 5 μM khellin (lane 6), 5 μM khellin plus UV-A light (lane 7), without PT (lane 8), ROS membranes (lane 9). Similar results were obtained in two other experiments. Quantification of the radioactivity of individual band from three experiments is given in Fig. 3A and 3B.

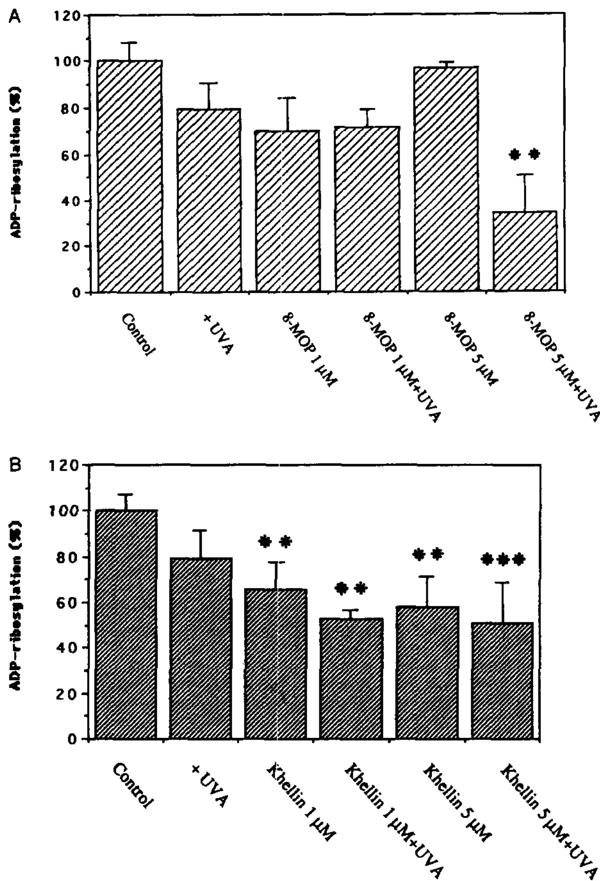

Fig. 3. (A) Effect of UV-A light, 8-MOP and a combination of the two on PT-dependent ADP-ribosylation of the α-subunit of Gi in HeLa cell membranes. The amount of ADP-ribosylation measured with 'ImageQuant' is expressed as the percentage of the control value obtained without UV-A light and drugs (mean values ± S.E.). ** $p < 0.01$ and *** $p < 0.001$, compared to control. (B) Effect of UVA light, khellin and a combination of the two on PT-dependent ADP-ribosilation of the α-subunit of Gi in HeLa cell membranes. The amount of ADP-ribosylation measured with 'ImageQuant' is expressed as the percentage of the control value obtained without UV-A light and drugs (mean values ± S.E.). ** $p < 0.01$ and *** $p < 0.001$, compared to control.

brane Giα. This assay is useful for monitoring the degree of ligand-induced G-protein activation [22,23].

Membranes, obtained from HeLa cells previously treated with drugs and UV-A light, as indicated in Section 2, were incubated with [^{32}P]NAD and PT. Gel electrophoresis followed by PhorphorImager analysis resulted in the predominant labelling of protein band with a molecular weight of 41 kDa (Fig. 2). Based on known specificity of the toxin and on electrophoretic mobility of ADP-ribosylated product the substrate is considered to be the α-subunit of Giα. Without PT no radiolabelling was observed (lanes 8 in Fig. 2). The amount of [^{32}P]ADP-ribosylation was determined by measuring the radioactivity of invidual bands in three experiments with 'ImageQuant'. Exposure to UV-A light alone decreases ADP-ribosylation but not significantly (Fig. 3). A significant inhibitory effect of 8-MOP on ADP-ribosyl-transferase activity is detectable only at the 5 μM concentration of the drug plus UV-A radiation (Fig. 3a). In contrast, khellin inhibits the enzyme even in dark conditions; UV-A light enhances the extent of inhibition, which reaches a maximum with the combination of khellin 5 μM and UV-A (Fig. 3b).

3.3. Increase of GTPase activity by cell treatment with khellin alone and plus UV-A light

Fig. 4a shows that GTPase activity is not affected by UV-A radiation or 8-MOP alone, or by a combination of the two. However, a significant increase in the enzymatic activity is seen when cells are treated with khellin, both alone and in combination with UV-A light, which is maximal at the higher concentration of the drug (Fig. 4b).

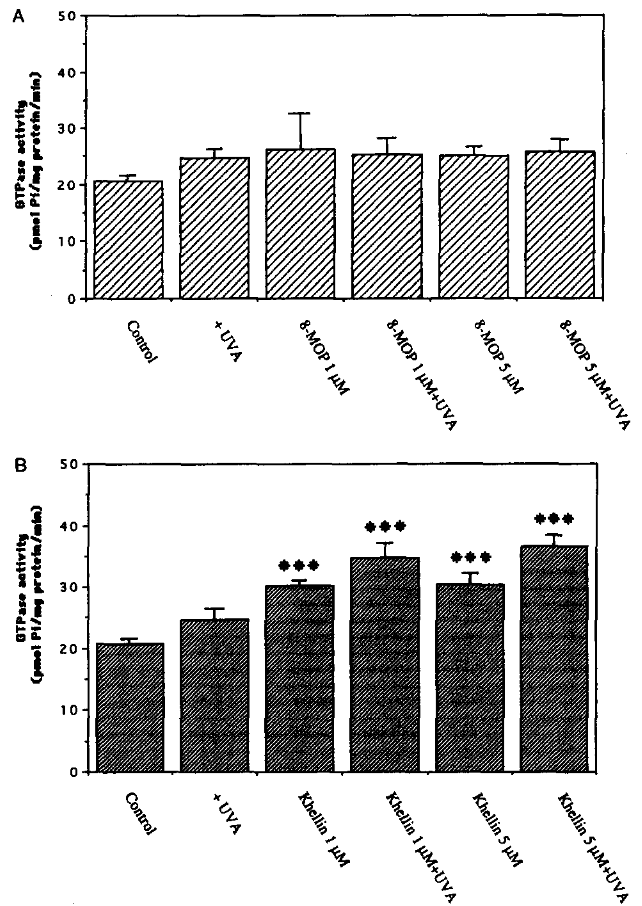

Fig. 4. (a) Effect of 8-MOP, UV-A light and a combination of the two on GTPase activity. Membranes were preparated as in Fig. 1A, and were assayed for GTPase activity as described in Section 2. Specific activity of GTPase is reported. Values represent the mean ± S.E., compared to control. (B) Effect of khellin, UV-A light and a combination of the two on GTPase activity. Membranes were preparated as in Fig. 1A, and were assayed for GTPase activity as described in Section 2. Specific activity of GTPase is reported. Values represent the mean ± SE. ** $p < 0.01$ and *** $p < 0.001$, compared to control.

4. Discussion

Relationships between adenylyl cyclase and UV-A, PUVA or psoralen alone have been detected in various cell types or tissues and in different experimental conditions [7].

Our studies indicate that, in HeLa cells, 8-MOP alone or in combination with UV-A light does not affect basal or stimulated adenylyl cyclase, suggesting that its main mode of action is the known effect of this psoralen on the EGF receptor [11,24] which is expressed in HeLa cells [9]. Although susceptible to interact with the 8-MOP receptor because of its structural similarity to 8-MOP, khellin (at 1 and 5 μM), both alone and in combination with UV-A light, inhibits NaF-stimulated adenylyl cyclase activity, but in the same experimental conditions, does not affect basal adenylyl cyclase. These results suggest that khellin does not interact with the catalytic subunit of adenylyl cyclase which is the reflection of basal enzyme activity and led us to investigate whether the inhibitory effect of khellin might be due to a receptor-coupled phenomenon probably requiring GTP-binding proteins.

It is well known that Gi protein mediates the inhibition of adenylyl cyclase in numerous cell types. Activation of heterotrimeric G protein by an agonist-occupied receptor results in an exchange of bound GDP for GTP in the α-subunit which then dissociates from the β-γ subunits' complex [21]. PT ADP-ribosylates only the unstimulated intact heterotrimeric G protein, whereas the free α-subunit is no longer susceptible to ADP-ribosylation [22,23]. We have demonstrated that cell treatment with khellin and UV-A light inhibits ADP-ribosylation of α-subunits of Gi catalyzed by PT and this inhibition is correlated with the inhibition of adenylyl cyclase activity. Thus our results indicate that khellin can trigger a dissociation of the α-subunit resulting in decreased ADP-ribosylation of Gi.

The inhibitory effect on ADP-ribosylation exerted by cell treatment with 8-MOP (at its higher concentration) in combination with UV-A light seems to be due to an aspecific interaction with the membrane, since it is not related to any effect on adenylyl cyclase activity. Support for this hypothesis comes from the experiments showing that cell treatment with 8-MOP, both alone and in combination with UV-A light, does not affect GTPase activity, which plays a central role in the phenomenon of receptor coupling with adenylyl cyclase [21]. In contrast, cell treatment with khellin increases GTPase activity both in the dark and plus UV-A light. It is therefore probable that a Gi protein is involved in the inhibitory effect of khellin on adenylyl cyclase activity.

Concerning HeLa cells, the following concluding remarks can be made. Khellin, a very weak DNA-photobinding compound, is able to exert its phototherapeutic activity probably on account of its effect on the adenylyl cyclase membrane system by means of inhibitory guanine nucleotide regulatory proteins. The receptor presumed to be involved in the process has no affinity (or the affinity cannot be detected) for 8-MOP which seems to act by means of other membrane sites [6].

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