A RAPID COLORIMETRIC MICROASSAY TO DETECT AGONISTS/ANTAGONISTS OF PROTEIN KINASE C BASED ON ADHERENCE OF EL-4.IL-2 CELLS

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A rapid, colorimetric, microassay for detection of agents which are known agonists/antagonists of protein kinase C (PKC) was developed, utilizing their effects on adherence of EL-4.IL-2 cells. Cells that were incubated with agents which are known inducers of PKC activation, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), mezerein and indolactam V, readily adhered to wells of 96 well microtiter plates within 1~2 hours, whereas cells incubated with the negative PKC activator, 4α-phorbol 12-myristate 13-acetate (4α-PMA), which is structurally related to PMA (4β-PMA), did not adhere. The adherent cells withstood repeated vigorous washings with tissue culture medium. Adherence of EL-4.IL-2 cells in the presence of PMA could be blocked by the addition of two known inhibitors of PKC, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride and staurosporine. Detection of the presence of adherent cells was accomplished by the addition of a tetrazolium salt to culture wells and determination of the remaining viable cells by scanning using a multiwell spectrophotometer (ELISA reader). The EL-4.IL-2 adherence assay meets several important criteria for use as a primary screen in the detection of potential PKC agonists/antagonists, *i.e.* its selectivity, simplicity, rapid performance through automation and reproducibility.

There is considerable interest regarding the crucial role that protein kinase C (PKC) plays in a multitude of cellular activation processes[1]. PKC, which is present in many tissues, is activated by diacylglycerol, a breakdown product of cell membrane-associated inositol phospholipids. The breakdown of these phospholipids can be induced by a number of extracellular signals (mitogens, growth factors, *etc.*) interacting with specific cell surface receptors. Phorbol esters (PE) appear to exert biological effects on cells *via* a direct activation of PKC through intercalation into the cell membrane, thereby substituting directly for diacylglycerol in the PKC activation process[2]. A number of these phorbol compounds, (phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 4-*o*-methyl PMA) have been used to study PKC activation in both normal and tumor cell lines, including studies on the activation of B lymphocytes[3~5] and T lymphocytes[6~8]. Studies on morphological and differentiative effects of PE's include the induction of aggregation and adherence of lymphocytes[9~11], increased locomotion and changes in shapes of human T lymphocytes[12,13], induction of adhesion and differentiation in human monoblastoid (U937)[14,15] and human promyelocytic leukemia (HL-60) cell lines[16], increased aggregation of neutrophils[17,18], stimulation of the production of interleukin-2 (IL-2) by normal mouse[19,20] and human lymphocytes[21] and by cultured EL-4 mouse thymoma cells[22]. SANDO *et al.*[23] reported the identification of phorbol ester receptors in EL-4 mouse thymoma cells and observed a phorbol ester (PMA)-induced adherence of an IL-2 producing subline of EL-4 cells. We were interested in determining if this PMA-induced adherence might be exploited in the development of a simple and rapid microassay system which would be capable of detecting agents whose mechanism of action might be similar to that of PMA (*i.e.* phorbol

binding and PKC activation), by observing the effects of these agents on adherence of EL-4.IL-2 cells. We report here a method by which the adherence of EL-4.IL-2 cells can be used to identify agents of certain classes which may serve to agonize and/or antagonize activation of PKC. The assay is rapid (6 hours total), and can be adapted to high volume screening by the use of microtiter plates and data readout using a colorimetric method and an automated plate reader.

Materials and Methods

Cells

EL-4.IL-2 cells (TIB 181), were obtained from American Type Culture Collection (Rockville, MD) and were maintained in culture by *in vitro* passage with tissue culture medium (TCM) (RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, 60 μg/ml L-glutamine, and 5×10^{-3} M 2-mercaptoethanol, Grand Island Biologicals Co., Grand Island, NY). Cells were passaged every 3 to 4 days. Viability was determined by trypan blue exclusion, and always exceeded 80%. For the adherence assay, cells were utilized within 15 passages of frozen stock cultures.

Reagents

PMA, PDBu, mezerein and 4α-phorbol 12-myristate 13-acetate (4α-PMA) were obtained from LC Services Corporation (Woburn, MA), 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride (H-7) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Staurosporine was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Indolactam V (−) and (+) isomers were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Stock solutions of PMA, PDBu, mezerein, 4α-PMA and staurosporine were prepared in DMSO. Indolactam V was prepared in ethanol. All solutions were stored at −70°C prior to use. Stock solutions of H-7 were prepared in sterile distilled water (10 mM) and kept at 4°C prior to use.

Adherence Assay

EL-4.IL-2 cells at various concentrations were cultured in 96-well, flat bottomed microtiter test plates (Nuncalon, Nunc A/S, Roskilde, Denmark) at 37°C in the presence or absence of test compounds and with the appropriate solvent controls. Incubation times were also varied. Following the incubation period, the contents of each well were removed by flicking the plate into a catch tray, and placing the plates inverted onto a paper towel in order to drain and blot the remaining draining wells. To each well, a volume of 100 μl of room temperature TCM was then added and the plates placed on a rotating platform shaker (Mini-Orbital Shaker, Bellco Biotechnology, Vineland, NJ) at setting "5" for 4 minutes. The plates were then rotated 180° and subjected to an additional 4 minutes of shaking. The plates were removed and their contents again removed as described, blotted, TCM was added to each well and the plates placed on the rotating shaker. This process was repeated for two additional wash cycles. The presence of viable cells was determined using a modified colorimetric method of MOSSMAN[24]. Briefly, following the final removal of well contents, a volume of 200 μl of TCM and 75 μl of MTT solution (2 mg/ml in TCM) was added to each well. Plates were incubated at 37°C for an additional 5 hours. Following incubation, the contents of the plates were again removed and the plates blotted onto paper towels. A volume of 200 μl of isopropyl alcohol was then added to each well in order to dissolve the resulting formazan crystals. The plates were read at 570 nm using a plate reader (Microplate Autoreader, Model EL-311, BIO-TEK Instruments, Inc., Winooski, VT).

Statistics

Triplicate OD readings were recorded for test and control wells and the mean and standard error determined for each data set.

Results

Optimization of EL-4.IL-2 Adherence Assay

Initial experiments were designed to optimize and standardize the PMA-induced adherence of EL-4.IL-2 cells in the development of a reproducible assay system. Fig. 1 shows results obtained when two concentrations of EL-4.IL-2 cells were incubated in flat-bottomed plates, with various concentrations of PMA or solvent control (0.0) for 1(A), 2(B) and 24(C) hours. Figs. 1(A) and 1(B) show that 1 or 2 hours incubations of EL-4.IL-2 cells yielded plates whose adherent cells were resistant to three cycles of washing, and which demonstrated a dose responsive adherence to PMA as measured by MTT metabolism. Incubation for 24 hours (Fig. 1(C)) resulted in a reduction of the total number of adherent cells as measured by the same tests. Solvent controls always consisted of the relative percentage of solvent contained in the highest concentration of compound tested and were consistently negative in inducing or inhibiting cell adherence. Fig. 2 shows that EL-4.IL-2 cells incubated in flat bottomed plates for 1 hour with 16 nM of PMA and washed three times following incubation consistently resulted in plates with greater numbers of adherent cells as measured by MTT absorbance, compared to identical conditions using U-bottom plates. While incubation of 5×10^5 EL-4.IL-2 cells per well resulted in the highest OD values under all conditions tested, a cell concentration of 2×10^5 cells was established for the standard assay in view of 1) need of fewer cells for the assay to be performed and 2) OD values which were still at least 74% of the highest OD values obtained. To determine the number of plate washes necessary to reduce the degree of potential non-specific attachment of non-PMA stimulated EL-4.IL-2 cells to an acceptable background OD value, unstimulated cells were incubated for 1 hour and subjected to various numbers of wash cycles. Results illustrated in Fig. 3 show that three wash cycles were sufficient to reduce non-specific attachment of EL-4.IL-2 cells to OD values of <0.001, with a corresponding PMA-stimulated attachment OD value of approximately 0.400. Increasing the number of washings did not appreciably reduce further EL-4.IL-2 attachment.

Effect of 4α-PMA on Adherence of EL-4.IL-2 Cells

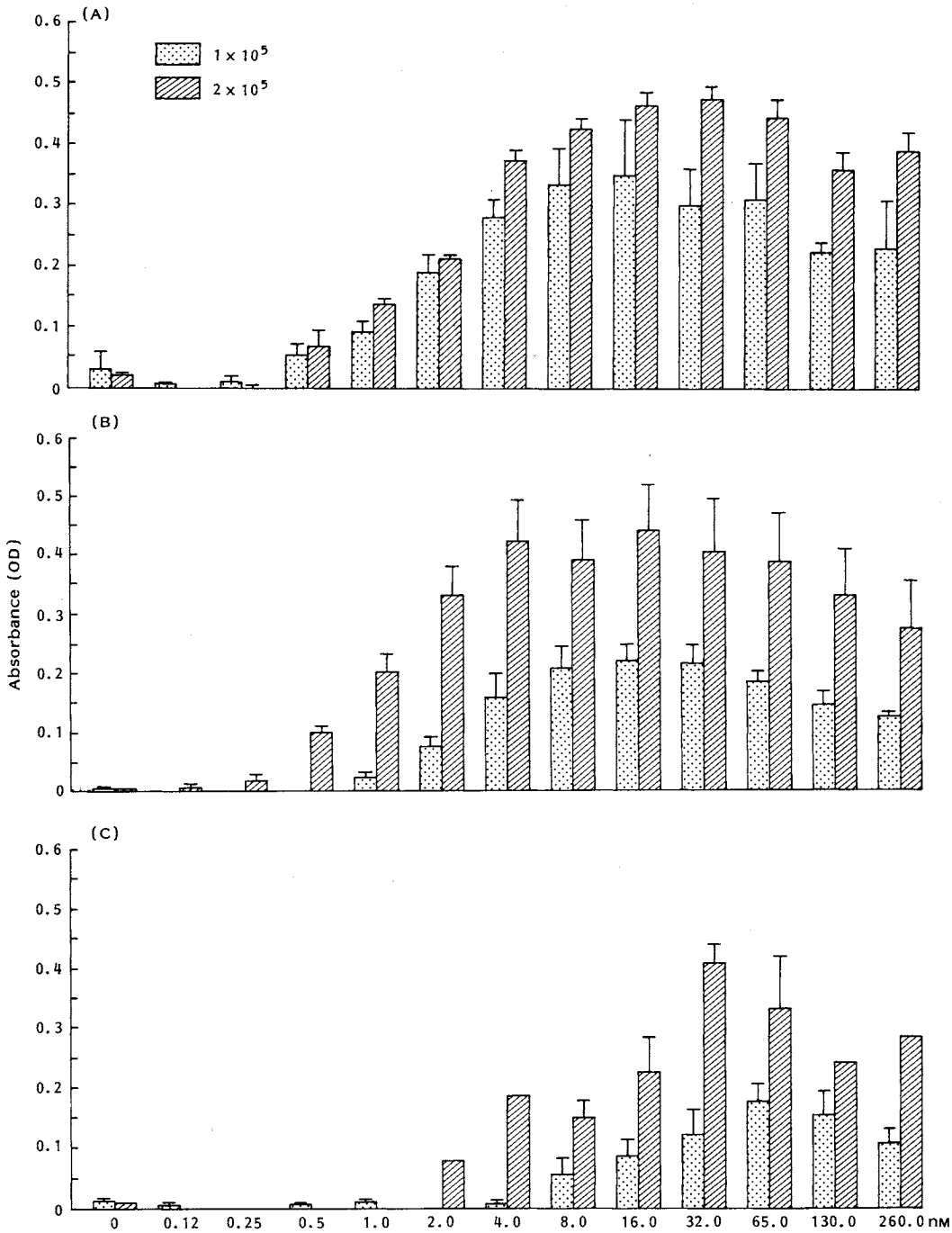
To determine if the structurally related compound 4α-PMA, which does not activate PKC, could induce a non-specific adherence of EL-4.IL-2 cells in our assay system, EL-4.IL-2 cells were incubated with various concentrations of 4α-PMA and 4β-PMA, and their effects on adherence evaluated. The results in Fig. 4 show that 4α-PMA did not induce non-specific adherence of EL-4.IL-2 cells, compared to the positive induction demonstrated by equivalent concentrations of 4β-PMA. This data indicates that adherence exhibited by EL-4.IL-2 cells could possibly be related to structural components of PMA which induce PKC activation.

Comparative Effects of PKC Activators Mezerein, PDBu and PMA on Adherence of EL-4.IL-2 Cells

EL-4.IL-2 cells were incubated for 1 hour with various concentrations of the PKC activators mezerein, PDBu, and PMA. The results in Fig. 5 show that all three agents were effective in the induction of a dose responsive adherence of EL-4.IL-2 cells following a 1-hour incubation. Both PMA and mezerein demonstrated peak adherence at 10 ng/ml (16 and 15 nM, respectively), while maximum cell adherence for PDBu occurred at 80 ng/ml (160 nM). These results indicate that in addition to PMA, two PKC activators, mezerein and PDBu, induce the adherence of EL-4.IL-2 cells.

Fig. 1. Optimization of EL-4.IL-2 adherence assay.

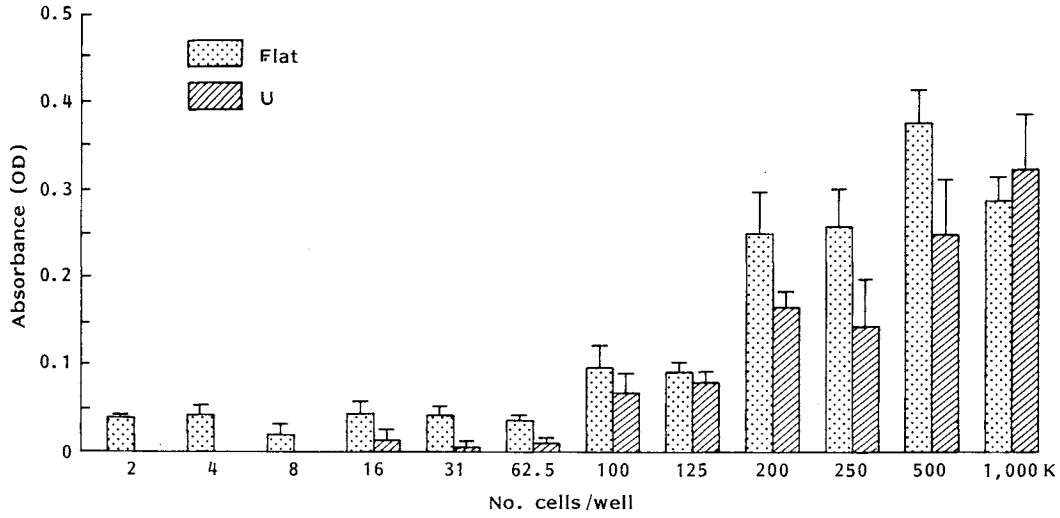
EL-4.IL-2 cells, 1×10^5 and 2×10^5 were incubated at 37°C for (A) 1 hour, (B) 2 hours or (C) 24 hours with or without various concentrations of PMA.



Plates were subjected to three cycles of warm (37°C) TCM washes and agitation as described in the text. A volume of 200 μl of TCM and 75 μl of MTT (2 mg/ml) was added to each of the wells and the plates incubated for an additional 5 hours. Supernatants were removed and isopropyl alcohol added to each well. Plates were read on an ELISA plate reader at a wavelength of 570 nm. Results are expressed as mean absorbance values of triplicate wells, $\pm$SE.

Fig. 2. Effect of cell concentration and well type on PMA-induced adherence of EL-4.IL-2 cells.

Various concentrations of EL-4.IL-2 cells were incubated with 16 nM PMA in either flat or U-bottomed plates for 1 hour at 37°C, and the plates processed, stained and absorbances determined as described in the text.



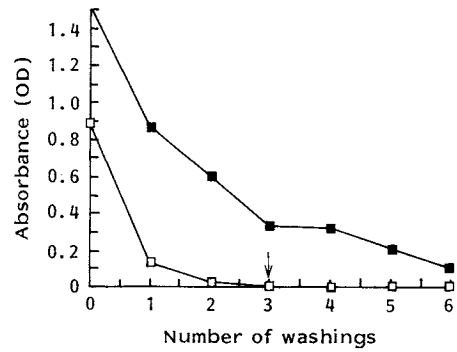
Results are expressed as mean absorbance values of triplicate wells, ±SE.

Effects of Indolactam V on Adherence of EL-4.IL-2 Cells

Indolactam V, the basic structural nucleus of two compounds which activate PKC (lyngbyatoxin A and the teleocidins) was evaluated for its ability to induce adherence of EL-4.IL-2 cells. The (−) isomer of indolactam V which has been reported to be active in adherence of HL-60 cells (FUJIKI *et al.*[25]) and the (+) isomer which is commonly used as the negative control were tested in the adherence assay. Fig. 6 shows that the (−) isomer induced a significant dose responsive adherence of EL-4.IL-2 cells, with peak responsiveness occurring at 10.0 μM, following a 1 hour incubation. The (+) isomer was effective in inducing adherence, but only at concentrations which were approximately 10-fold higher and only within a narrow concentration range. These results show that the (−) isomer of indolactam V which is structurally unrelated to the phorbol esters but which is a potent activator of PKC can similarly induce the adherence of EL-4.IL-2 cells in our system.

Fig. 3. Effect of plate washings on the non-specific adherence of non-adherent EL-4.IL-2 cells.

EL-4.IL-2 cells (2.0×10^5) were added to wells of microtiter plates in the presence (■) and absence (□) of PMA.



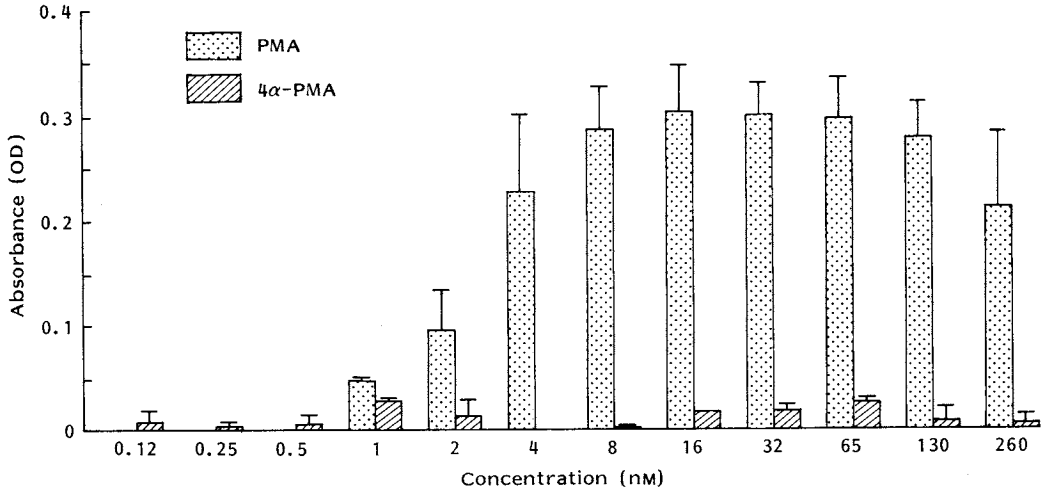
Plates were incubated for 1 hour followed by the indicated numbers of plate washings. Plates were stained and absorbances determined as described in the text. Results are expressed as mean absorbance values of triplicate wells.

Effect of PKC Inhibitors H-7 and Staurosporine on Adherence of EL-4.IL-2 Cells

If the adherence of EL-4.IL-2 cells is linked to the activation of PKC, then such adherence would be

Fig. 4. Effect of 4α-PMA on adherence of EL-4.IL-2 cells.

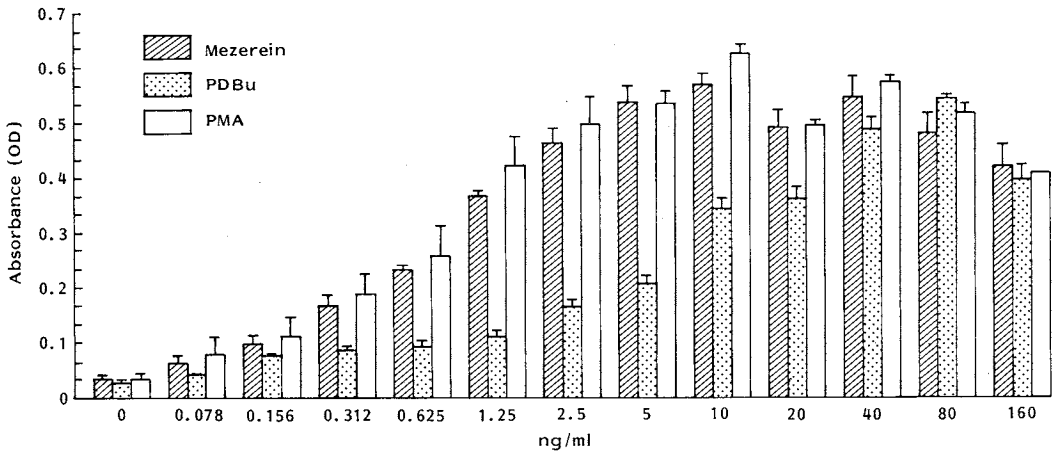
Various concentrations of PMA and 4α-PMA were added to wells of microtiter plates containing EL-4.IL-2 cells and incubated at 37°C for 1 hour.



Plates were processed, stained and absorbances determined as described in the text. Results are expressed as mean absorbance values of triplicate wells, ±SE.

Fig. 5. Comparative effects of the PKC activators mezerein, PDBu, and PMA on adherence of EL-4.IL-2 cells.

Various concentrations of mezerein, PDBu, or PMA were added to wells of microtiter plates containing EL-4.IL-2 cells and incubated at 37°C for 1 hour.

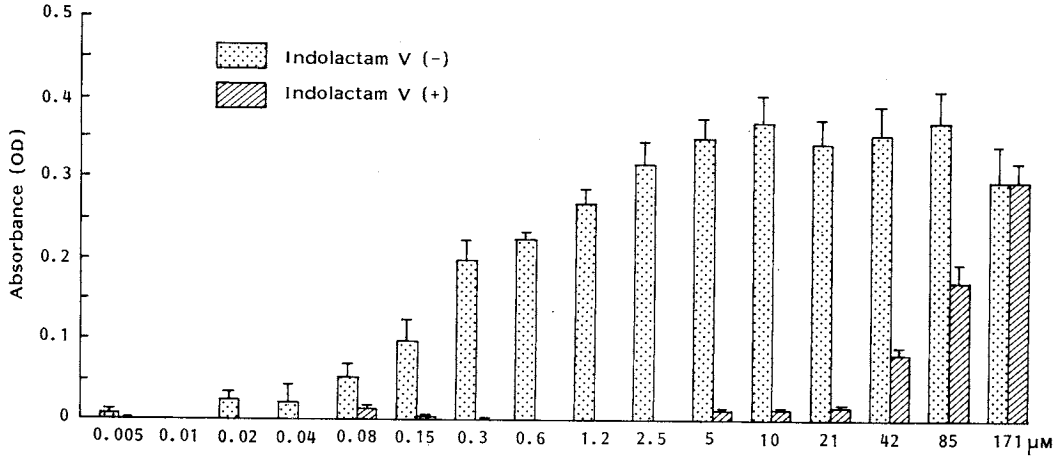


Plates were processed, stained and absorbances determined as described in the text. Results are expressed as mean absorbance values of triplicate wells, ±SE.

expected to be blocked by agents which serve to inhibit the activation of PKC. Two known inhibitors of PKC, H-7 and staurosporine were evaluated as to their effects on adherence of EL-4.IL-2 cells. Compounds were tested under identical conditions, using 2×10^5 EL-4.IL-2 cells per well in the presence of PMA (32 nM final concentration) with or without the inhibitors. Both compounds were shown to be non-toxic for

Fig. 6. Effect of indolactam V on adherence of EL-4.IL-2 cells.

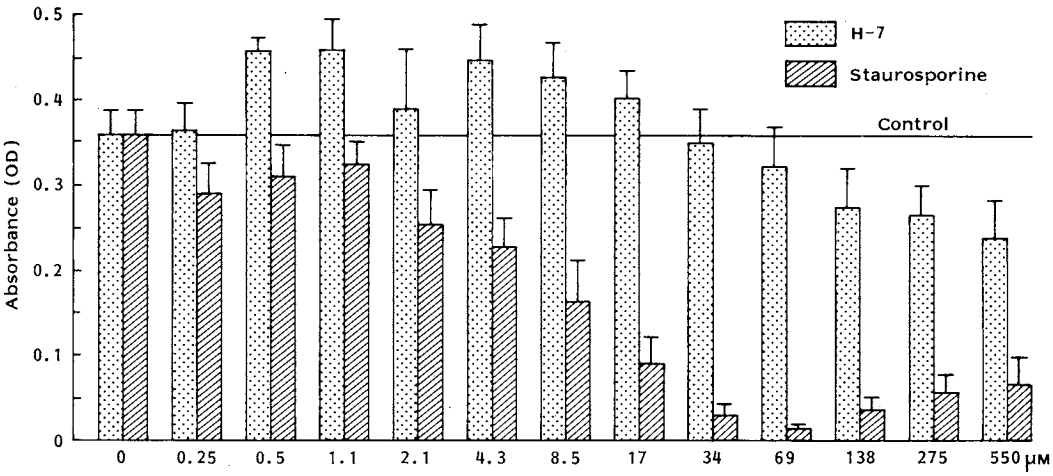
The two isomers of indolactam V, (−) and (+), were added at varying concentrations to EL-4.IL-2 cells and incubated for 1 hour at 37°C.



Plates were processed, stained and absorbances determined as described in the text. Results are expressed as mean absorbance values of triplicate wells, ±SE.

Fig. 7. Effect of the PKC inhibitors H-7 and staurosporine on adherence of PMA stimulated EL-4.IL-2 cells.

EL-4.IL-2 cells were incubated in the presence of 32 nM PMA with various concentrations of H-7 or staurosporine for 1 hour at 37°C.



Plates were processed, stained and absorbances determined as described in the text. Results are expressed as mean absorbance values of triplicate wells, ±SE.

EL-4.IL-2 cells following a 2-hour incubation, as measured by trypan blue exclusion and metabolism of MTT (data not shown). Fig. 7 shows that H-7 suppressed the PMA-induced adherence of EL-4.IL-2 cells at 138, 275, and 550 μM, however, lower concentrations (0.5 ~ 17 μM) induced adherence above the control values. Staurosporine inhibited the PMA-induced adherence of EL-4.IL-2 cells in a dose responsive manner, with maximum inhibition of the adherence occurring at 43 nM. H-7 and staurosporine, themselves, did not

significantly induce the adherence of EL-4.IL-2 cells in the absence of PMA (data not shown). These results show that inhibitors of PKC activation also serve to inhibit the adherence of PMA stimulated EL-4.IL-2 cells.

Discussion

Recently, OSADA *et al.*[26] reported a cellular based screening method for detection of inhibitors of PKC, utilizing a "bleb forming assay" by which activators of PKC induced the formation of "blebs" on the surface of K562, a human chronic myeloid leukemia cell line. The assay was reported to be selective and easy to perform, however, their method required a time consuming microscopic examination of the tumor cells to detect appearance of the "blebs", and did not include provisions for measuring cellular viability or the adaptability for automation.

Our initial observations and reports of others[23,27] on the induction of adherence of EL-4 cells by PMA has lead us to the development of a cellular-based microassay which is capable of detecting compounds which are known agonists/antagonists of PKC. The assay is simple, fast and easy to perform (automated). Stimulated, adherent EL-4.IL-2 cells were extremely resistant to repeated cycles of washing with TCM and those cells which remained attached to wells of microtiter plates metabolized MTT efficiently, an indication of a high degree of viability. Non-specific adherence of non-stimulated cells was consistently low, as evidenced by the lack of MTT metabolizing cells on the surface of the microtiter wells. Since compounds at high concentrations could exert cytotoxic effects on cells and thus prevent their attachment, parallel plates which were not subjected to washes were always included in each assay in order to detect cytotoxic effects, *via* MTT metabolism and trypan blue exclusion.

Several important aspects regarding the performance of the assay were revealed during its development. We found that consistent results were obtained if the EL-4.IL-2 cells which were to be used for the assay were in the log phase of growth and within $1 \sim 15$ passages of the ATCC stock culture. Older cultures (*i.e.* >15 passages) tended to display reduced adherence characteristics when stimulated with PMA (data not shown). Although we did not investigate the specific cause of this phenomenon, it may be due, in part, to a selection of a cell type which is resistant to PMA effects[23]. In addition, warmed (37°C) medium was always used for the washings, since cold medium tended to induce cell detachment.

Our assay does not directly measure PKC enzyme activity, however, several lines of evidence strongly support our premise that this assay may be a useful adjunct in the detection of potential agonists/antagonists of PKC. Early studies by KRAFT *et al.*[28] documented a decrease in cytosolic calcium phospholipid-dependent protein kinase (PKC) activity following treatment of EL-4 thymoma cells with phorbol esters. Subsequent studies with EL-4 sublines and other cell types have confirmed the observed translocation of PKC activity from the cytosol to cell membrane[29] and this phenomenon is now regarded as a generally accepted mechanism of PKC activation by phorbol esters and other PKC activators[1].

Both PDBu and mezerein, compounds which are structurally related to PMA and which act as PKC activators, induced a dose responsive adherence of EL-4.IL-2 cells as measured by our assay. Even more compelling evidence linking EL-4.IL-2 adherence with PKC activation is supported by our results which verify the exquisite selectivity of phorbol receptors for 4β- but not 4α-PMA in the induction of PKC activation, as measured by cell adherence in our assay as well as in other reported systems[30].

An interesting aspect of our study included the analysis of the compound indolactam V, which is structurally unrelated to PMA, but with reported PKC activation. Both isomeric forms of indolactam V induced the adherence of EL-4.IL-2 cells, with the (+) form exhibiting approximately 100-fold less potent (μg/ml) activity compared to the (−) form. Our results are consistent with studies of FUJIKI *et al.*[25] who reported similar concentration effects of the indolactams as measured by adhesion of HL-60 cells.

We also tested two known inhibitors of PKC, which demonstrated varying degrees of potency in their ability to inhibit the adherence of EL-4.IL-2 cells. Our results were consistent with those of others, using other test systems, who have reported similar rankings of PKC inhibitory activity (*i.e.* staurosporine $\geq$H-7)[31~33]. The apparent stimulatory effect of the adherence of PMA stimulated EL-4.IL-2 cells which we observed at concentrations of H-7 less than 8.5 μM cannot be fully explained, however, concentrations of H-7 which are routinely reported to inhibit cellular based, but not isolated enzyme PKC assays, are commonly in the range of 20 μM to 500 μM[11,18,26], similar to the inhibition range we report here. The

concentrations of H-7 and staurosporine used in our experiments were shown not to be toxic under the conditions used in our assay system and was verified using MTT metabolism and trypan blue exclusion. The results of our study thus provide reasonable evidence for a positive role for PKC activation in the induction of EL-4.IL-2 cell adherence. While our adherence assay can be utilized as a rapid and easy "first pass" method for screening unknown compounds for potential PKC agonism/antagonism, final determination of any compound's potential effects on PKC activity should be confirmed using the actual PKC enzyme assay.

The EL-4.IL-2 adherence assay may prove useful as a high volume screening assay, not only for potential PKC agonists/antagonists, but as a tool to identify potential agonists/antagonists of IL-2 production. We observed in our studies that the optimal concentrations of PMA used to induce adherence of the EL-4.IL-2 cells correspond to those which are commonly used to induce IL-2 production ($10 \sim 20$ ng/ml or $16 \sim 32$ nM)[19].

In summary, the EL-4.IL-2 adherence assay is a relatively simple, fast and reproducible automated bioassay which may prove useful in the identification of PKC agonists/antagonists. Further studies which will demonstrate the utility of the adherence assay for analysis of crude extracts of marine organisms for potential PKC agonism/antagonism will be the subject of a future paper.

Acknowledgments

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References

1) NISHIZUKA, Y.: Studies and perspectives of protein kinase C. Science 233: 305~312, 1986
2) CASTAGNA, M.; Y. TAKAI, K. KAIBUCHI, K. SANO, U. KIKKAWA & Y. NISHIZUKA: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847~7851, 1982
3) CHEN, Z. Z.; K. M. COGGESHALL & J. C. CAMBIER: Translocation of protein kinase C during membrane immunoglobulin-mediated transmembrane signaling in B lymphocytes. J. Immunol. 136: 2300~2304, 1986
4) RANSOM, J. T. & J. C. CAMBIER: B-cell activation. VII. Independent and synergistic effects of calcium and diacylglycerol on membrane potential and I-A expression. J. Immunol. 136: 66~72, 1986
5) KLAUS, G. G.; M. K. BIJSTERBOSCH, A. O'GARRA, M. M. HARNETT & K. P. RIGLEY: Receptor signalling and crosstalk in B lymphocytes. Immunol. Rev. 99: 19~38, 1987
6) WIESS, A.; J. IMBODEN, K. HARDY, B. MANGER, C. TERHORST & J. STOBO: The role of T3/antigen receptor complex in T-cell activation. Annu. Rev. Immunol. 4: 593~619, 1986
7) ISAKOV, N.; M. I. MALLY, W. SCHOLZ & A. ALTMAN: T-lymphocyte activation: The role of protein kinase C and the bifurcating inositol phospholipid signal transduction pathway. Immunol. Rev. 95: 89~111, 1987
8) DAVIS, L. S. & P. E. LIPSKY: T cell activation induced by anti-CD3 antibodies requires prolonged stimulation of protein kinase C. Cell. Immunol. 118: 208~221, 1989
9) PATARROYO, M. & M. JONDAL: Phorbol ester-induced adhesion (binding) among human mononuclear leukocytes requires extracellular Mg^{++} and is sensitive to protein kinase C, lipoxygenase and ATPase inhibitors. Immunobiology 170: 305~319, 1985
10) ROTHLEIN, R. & T. A. SPRINGER: The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. 163: 1132~1149, 1986
11) PHATAK, P. D.; C. H. PACKMAN & M. A. LICHTMAN: Protein kinase C modulates actin conformation on human T lymphocytes. J. Immunol. 141: 2929~2934, 1988
12) HASTON, W. S.; J. M. SHIELDS & P. C. WILKINSON: Lymphocyte locomotion and attachment on two-dimensional surfaces and in three-dimensional matrices. J. Cell Biol. 92: 747~752, 1982
13) WILKERSON, P. C.; J. M. LACKIE, W. S. HASTON & L. N. ISLAM: Effects of phorbol esters on shape and locomotion of human blood lymphocytes. J. Cell Science 90: 645~655, 1988
14) WIEDERHOLD, M. D.; K. M. ANDERSON & J. E. HARRIS: Labeling of lipids and phospholipids with ^{3}H-arachidonic acid and the biosynthesis of eicosanoids in U937 cells differentiated by phorbol ester. Biochem. Biophys. Acta 959: 296~304, 1988
15) HASS, R.; L. KÖHLER, W. REHFELDT, V. LEßMANN, W. MÜLLER, K. RESCH & M. GOPPELT-STRUEBE: Alterations in glycosylation and lectin pattern during phorbol ester-induced differentiation of U937 cells. Cancer Res. 50:

323～327, 1990

16) HARRIS, P. & P. RALPH: Human leukemic models of myelomonocytic development: A review of the HL-60 & Y937 cell lines. J. Leukocyte Biol. 37: 407～422, 1985

17) O'FLAHERTY, J. T.; L. R. DECHATELET, C. E. MCCALL & D. A. BASS: Neutrophil aggregation: Evidence for a different mechanism of action by phorbol myristate acetate. Proc. Soc. Exp. Biol. Med. 165: 225～232, 1980

18) MILLER, D. K. & S. SADOWSKI: Development of a high capacity microassay for measurement of neutrophil adhesion. J. Immunol. Methods 106: 37～47, 1988

19) FARRAR, J. J.; S. B. MIZEL, J. FULLER-FARRAR, W. L. FARRAR & M. L. HILFIKER: Macrophage-independent activation of helper T cells. 1. Production of Interleukin 2. J. Immunol. 125: 793～798, 1980

20) FULLER-FARRAR, J.; M. L. HILFIKER, W. L FARRAR & J. J. FARRAR: Phorbol myristic acetate enhances the production of Interleukin 2. Cell Immunol. 58: 156～164, 1981

21) STADLER, B. M.; S. F. DOUGHERTY, J. J. FARRAR & J. J. OPPENHEIM: Relationship of cell cycle to recovery of IL2 activity from human mononuclear cells, human and mouse T cell lines. J. Immunol. 127: 1936～1940, 1981

22) FARRAR, J. J.; J. FULLER-FARRAR, P. L. SIMON, M. L. HILFIKER, B. M. STADLER & W. L. FARRAR: Thymoma production of T cell growth factor (Interleukin 2). J. Immunol. 125: 2555～2558, 1980

23) SANDO, J. J.; M. L. HILFIKER, M. J. PIACENTINI & T. M. LAUFER: Identification of phorbol ester receptors in T-cell growth factor-producing and -nonproducing EL4 mouse thymoma cells. Cancer Res. 42: 1676～1680, 1982

24) MOSSMAN, T.: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55～63, 1983

25) FUJIKI, H.; M. SUGANUMA, M. NAKAYASU, T. TAHIRA, Y. ENDO, K. SHUDO & T. SUGIMURA: Structure-activity studies on synthetic analogues (indolactams) of the tumor promoter teleocidin. Gann 75: 866～870, 1984

26) OSADA, H.; J. MAGAE, C. WATANABE & K. ISONO: Rapid screening method for inhibitors of protein kinase C. J. Antibiotics 41: 925～931, 1988

27) STOECK, M.; R. LEES, G. PANTALEO & H. R. MACDONALD: Comparison of phorbol-12-myristate-13-acetate and dioctanoyl-sn-glycerol in the activation of EL4/6.1 thymoma cells. J. Cell. Physiol. 138: 541～547, 1989

28) KRAFT, A. S.; W. B. ANDERSON, H. L. COOPER & J. J. SANDO: Decrease in cytosolic calcium/phospholipid dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J. Biol. Chem 257: 13193～13196, 1982

29) KRAMER, C. M. & J. J. SANDO: Substrates for protein kinase C in cytosol of EL4 mouse thymoma cells. Cancer Res. 46: 3040～3045, 1986

30) BLUMBERG, P. M.: *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters. Part 1. CRC Crit. Rev. Toxicol. 8: 153～197, 1980

31) TAMAOKI, K.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid-calcium dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397～402, 1986

32) HIDAKA, H.; M. INAGAKI, S. KAWAMOTO & Y. SASAKI: Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23: 5036～5041, 1984

33) KAWAMOTO, S. & H. HIDAKA: 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem. Biophys. Res. Commun. 125: 258～264, 1984