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# Protein kinase C isotype expression and regulation of lymphoid cell motility

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## SUMMARY

Lymphocyte migration into inflammatory sites involves a change from a spherical, non-motile phenotype to an irregular, constantly shape-changing, motile phenotype. We have previously shown that lymphocytes are maintained in the non-motile state by the constitutive activity of protein kinase C (PKC). In this paper we have attempted to identify the PKC isotype which regulates these morphological changes by three different approaches. (a) Motile and non-motile T-cell lines were compared for expression of the $\alpha$, $\beta$I, $\beta$II, $\gamma$, $\delta$, $\varepsilon$, $\eta$, $\zeta$ and $\theta$ isotypes by Western blotting. There was no obvious correlation of isotype expression with motility. (b) Two different PKC inhibitors, one specific for classical isotypes, Go6976 and the other GF109203X, which inhibits both classical and non-classical isotypes were compared for induction of motility in non-motile lymphocytes. Only GF109203X induced motility implying that a non-classical isotype is involved. (c) Non-motile lymphocytes were chronically treated with the PKC activator bryostatin and the time courses of induction of motility and downregulation of PKC isotypes were compared. Induction of motility correlated better with downregulation of $\varepsilon$, $\eta$ and $\theta$ than with $\alpha$ or $\beta$. It is concluded that the data fit best with the involvement of a non-classical PKC isotype in regulating lymphocyte motility although no association with a particular isotype was found.

## INTRODUCTION

Lymphocytes are professionally migratory cells, constantly moving from the bloodstream into the tissues and back again via lymphatics in their constant search for antigen. In the bloodstream, lymphocytes are predominantly rounded cells incapable of locomotion but adhesion to specialized endothelium in lymph nodes or activated endothelium in inflammatory sites results in a switch to the motile lymphocyte phenotype. This is characterized by a constantly changing, irregular cell shape resulting from rapid changes in actin polymerization.[1]

The induction of the motile phenotype is essential for the lymphocyte to move between endothelial cells and through the extravascular tissues. Despite the importance of this phenomenon very little is known about the biochemical mechanisms which induce motility or maintain it. A wide range of agents has been reported to induce motility, including IL-2, IL-15[2] and many of the chemokines, which act in a subset-specific manner. The very diversity of motility-inducing agents implies the existence of a final common pathway.

Recently we have shown that inhibition of protein kinase C (PKC) is very effective in inducing the motile phenotype in a large proportion of peripheral blood lymphocytes (PBLs). This effect was not subset-specific and could be mediated either with a specific PKC inhibitor of the bisindolylmaleimide type or by downregulation of PKCs by chronic treatment with bryostatin.[3]

Induction of motility by (PKC) inhibition was observed both with primary lymphocyte isolates and with a non-motile variant of the MOLT-4 T cell line.[3] In contrast, with the spontaneously motile variant of MOLT-4 ('motile MOLT-4'), acute treatment with PKC activators caused inhibition of motility, an effect which could be overridden with bisindolylmaleimides. Therefore, in these experimental systems lymphocyte motility can be regulated at will — PKC inhibition induces the motile phenotype and PKC activation induces the non-motile phenotype.

The PKC family is comprised of at least 10 isotypes which differ in their cellular distributions, subcellular localizations and regulatory mechanisms.[4] With respect to the latter, the PKC isotypes are classified into two main groups. The classical PKCs consist of isotypes $\alpha$, $\beta$I, $\beta$II, and $\gamma$ which are calcium dependent and activatable by phorbol esters. The non-classical isotypes encompass either the 'novel' isotypes $\delta$, $\varepsilon$, $\eta$, and $\theta$ which are activatable by phorbol esters but are calcium independent or the 'atypical' isotypes $\zeta$, $\lambda$, $\mu$, and $\iota$ which are calcium independent and not activatable by phorbol esters. In the current study we endeavoured to identify the PKC isotype(s) which regulates lymphocyte motility. The candidate isotype(s) should be (a) expressed in non-motile cells which acquire the motile phenotype on bisindolylmaleimide treatment

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and (b) expressed in spntaneously motile lines which acquire the non-motile phenotype after treatment with a PKC activator. To this end we have compared PKC isotype expression in a panel of motile and non-motile variants of T-cell lines.

## MATERIALS AND METHODS

*Cells*

MOLT-4, CCRF-CEM and BW5147.3 lymphoid cell lines were obtained from ECACC (Porton Down, UK) and maintained in growth medium (RPMI-1640 with 10% fetal calf serum (FCS), 50 IU/ml penicillin and 50 $\mu$g/ml streptomycin) at 37°. Motile and non-motile variants of MOLT-4 were isolated as described previously.[3] CCRF-CEM, EL4-B and BW5147.3 were inherently highly shape changed and so no further selection was necessary.

The establishment of the other BW5147 variants has been described previously.[1] BW5147.0 and BW5147.6 were predominantly round, whereas BW-O-Li1 and BW-O-Li3B were mainly of the shape-changed phenotype. Human peripheral blood lymphocytes (PBL) were purified as described earlier[5] and a T-cell-enriched fraction was obtained by anti-CD14 and anti-CD19 depletion of monocytes and B lymphocytes with magnetic beads (Dynal, Oslo, Norway).

*Chemicals*

The bisindolylmaleimide (GF109203X) PKC inhibitor was purchased from Calbiochem Novabiochem (Nottingham, UK) and the structurally related compound, indolocarbazole (Go6976)[6] was synthesized by Ferring Research Institute (Southampton, UK). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (Poole, UK) and bryostatin-1 from LC laboratories (Boston, MA).

*Shape-change assay*

In 96-well cluster plates, 50 $\mu$l of cell suspension in growth medium ($5 \times 10^6$/ml) was added to 50 $\mu$l of test reagent. In the case of procedures requiring two types of compounds e.g. PMA plus GF109203X, the inhibitor was added to the cell suspension just prior to the activator and the mixture incubated for 1 hr at 37°.

Following treatment cells were fixed in a final concentration of 3·7% formaldehyde/phosphate-buffered saline (PBS) and then assessed microscopically for the proportion of irregular, shape-changed cells.[5] Each experiment was set up in triplicate and results expressed as the mean percentage shape-changed cells ± standard deviation (SD).

*Western blotting*

The cell suspension ($5 \times 10^6$ cells) was washed twice and then resuspended in 100 $\mu$l reducing sodium dodecyl sulphate (SDS) loading buffer[7] and boiled for 5 min. Following polyacrylamide gel electrophoresis (PAGE) and transblot to 0·2 $\mu$m nitrocellulose, blots were blocked with 3% milk powder in PBS, for 1 hr at room temperature.[3] Blots were probed with either (a) mouse PKC $\alpha$ and $\beta$ monoclonal antibodies (Seikagaku Corporation, Tokyo, Japan) diluted to 50 ng/ml, (b) rabbit PKC $\eta$, $\beta$I and $\beta$II polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at concentrations between 25–100 ng/ml, (c) affinity purified rabbit PKC $\gamma$, $\delta$, $\varepsilon$ and $\zeta$ polyclonal antibodies (Gibco BRL, Paisley, UK) diluted to concentrations between 25–250 $\mu$g/ml or (d) mouse PKC $\theta$ monoclonal antibody (Affinti Research Products Limited, Exeter, UK) diluted to 80 ng/ml. All antibodies were diluted in PBS/0·05% Tween 20 and incubated for 2 hr at room temperature, except the mouse monoclonal antibodies which were incubated at 4° overnight. The second antibodies, Goat anti-rabbit IgG horseradish peroxidase (HRP) (Biorad Laboratories, Hemel Hempstead, UK) diluted to 1 : 10 000 or goat anti-mouse (IgG) HRP (Sigma Immunochemicals, Poole, UK) diluted to 1 : 3000, were incubated for at least 2 hr at room temperature. Immunoblots were developed by the ECL detection method (Amersham Life Sciences, Amersham, UK).

*Transfection and expression of PKC $\beta$*

PKC $\beta$I and $\beta$II cDNAs were obtained from Dr Peter Parker (Imperial Cancer Research Fund, London, UK) and cloned into the mammalian transfection vector pcDNA1neo (R&D systems, Oxford, UK). BW5147.3 cells were electroporated with a Bio-rad Gene Pulser apparatus at 250 V (960 $\mu$F) and selected after 48 hr recovery period with 2 mg/ml G418 (Sigma, UK) in normal growth medium. Surviving cells were cloned and screened for PKC $\beta$ expression by Western blotting. The highest expressing clones were then selected.

*Downregulation of PKC*

Bryostatin was added to the non-motile cells at a concentration of 50 nM in their normal growth medium and then incubated for 2, 18 or 44 hr at 37°. After treatment, a 200 $\mu$l volume of the cell suspensions was retained for the shape-change assay. The remainder was prepared for Western blot analysis as above.

## RESULTS

If activation of a particular PKC isotype maintains lymphocytes in the non-motile state then spontaneously motile T-lymphocyte cell lines may either (a) lack that isotype or (b) lack the means to activate it. Conversely, any non-motile lines which can be induced to motility must express that isotype and have the means to activate it. Because we do not know what the activation mechanisms are, our starting point has been to compare PKC isotype expression in a range of motile and non-motile T lymphoid lines. Initially, classical isotype expression was studied.

### Classical PKC isotype expression in motile and non-motile T lymphoid lines

Western blots for PKC $\alpha$, $\beta$I and $\beta$II expression are shown in Fig. 1 and summarized in Table 1. From these data it can be concluded that there is no simple correlation between expression of either PKC $\alpha$, $\beta$I or $\beta$II and motility. PKC $\gamma$ could not be detected in any of the cell lines (data not shown).

Some of the motile cell lines such as MOLT-4 and CCRF-CEM can be converted to the non-motile phenotype by acute treatment with PMA whilst others cannot (Table 2). Therefore motile MOLT-4 and CCRF-CEM must express the isotype which maintains the non-motile state. This cannot be $\alpha$ (CCRF-CEM does not express it), but could be $\beta$I or $\beta$II, as both lines express them. However, the motile lines EL4.B and BW5147.Li3B cannot be restored to the non-motile phenotype by PMA activation although both express $\beta$I and $\beta$II (Table 2). Further evidence against a role for PKC $\beta$ was obtained by

 

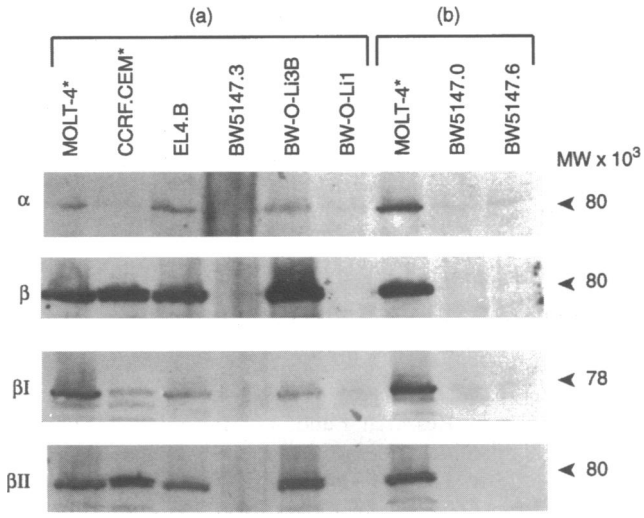


**Figure 1.** Expression of classical PKC isotypes in (a) motile and (b) non-motile T-cell lines as detected by Western blotting. * human cell lines, the remainder are murine.

transfection of PKC βI or βII into the motile BW5147.3 cells which normally do not express PKC β. Like the wild-type cells, BW5147.3 transfectants expressing either βI or βII (Fig. 2) retained the motile phenotype with 60–80% shape-changed cells and this was not altered by treatment with 25 nM PMA for up to 1 hr.

The tentative conclusion at this stage is that activation of a classical isotype is not responsible for maintaining the non-motile state. This raises the possibility that it is a non-classical isotype. In this case, in non-motile lymphocytes, motility should be induced by a PKC inhibitor which acts on non-classical isotypes but not by an inhibitor whose effects are restricted to classical isotypes. This is tested in the next section.

**Table 1.** Summary of PKC isotype expression in motile and non-motile T-cell lines and the effects of PMA and GF109203X on cell shape change

| | α | βI | βII | γ | δ | ε | η | θ | PMA effects† |
|---|---|---|---|---|---|---|---|---|---|
| *Motile* | | | | | | | | | |
| MOLT-4* | + | + | + | – | – | + | + | + | + |
| CCRF-CEM* | – | + | + | – | – | – | + | + | + |
| EL4.B | + | + | + | – | + | + | + | + | – |
| BW5147.3 | – | – | – | – | + | + | – | – | – |
| BW-O-Li3B | + | + | + | – | + | + | + | + | – |
| BW-O-Li1 | – | – | – | – | + | + | – | – | – |
| *Non-motile* | | | | | | | | | GF109203X effects‡ |
| MOLT-4* | + | + | + | – | – | + | + | + | + |
| BW5147.0 | ± | – | – | – | + | + | – | – | + |
| BW5147.6 | ± | – | – | – | + | + | – | – | – |

* Human cell lines, the remainder are murine.

† Acute PMA treatment resulted in reversion to a rounded phenotype (+) or had not effect (–) on the shape changed morphology of the motile cells.

‡ Acute GF109203X treatment induced a shape changed phenotype (+) or had no effect (–) on non-motile cells.

**Table 2.** PMA effects on motile cell lines as measured in a shape change assay. Percentage of shape-changed cells (mean ± SD)

| | PMA concentration (nM) | | | | | |
|---|---|---|---|---|---|---|
| Cell line | 0 | 1·5 | 3·0 | 6·3 | 12·5 | 25 |
| MOLT-4 | 69 ± 5 | < 1 | < 1 | < 1 | < 1 | < 1 |
| CCRF-CEM | 57 ± 7 | < 1 | < 1 | < 1 | < 1 | < 1 |
| HUT.78 | 71 ± 8 | < 1 | < 1 | < 1 | < 1 | < 1 |
| U937 | 87 ± 6 | < 1 | < 1 | < 1 | < 1 | < 1 |
| EL4.B | 94 ± 2 | 83 ± 7 | 74 ± 8 | 81 ± 2 | 78 ± 1 | 75 ± 5 |
| BW5147.3 | 93 ± 2 | 96 ± 1 | 90 ± 1 | 92 ± 3 | 94 ± 3 | 90 ± 1 |
| BW-O-Li3B | 90 ± 4 | 87 ± 5 | 91 ± 3 | 90 ± 2 | 93 ± 3 | 86 ± 3 |

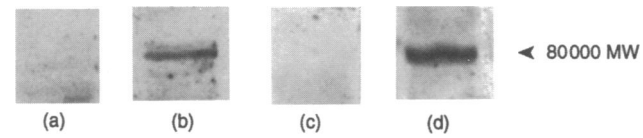


**Figure 2.** Expression of PKC β in wild type BW5147.3 cells (a, c) and following transfection with PKC βI (b) and PKC βII (d) by Western blotting.

### Induction of motility in non-motile lymphocytes by PKC inhibitors with different isotype specificities

Although limited information is available on isotype specificity, the bisindolylmaleimide GF109203X can inhibit at least some non-classical isotypes (δ and ζ) in addition to classical isotypes (α and β). In contrast, in the same study, a structurally related indolocarbazole (Go6976) inhibited only the classical isotypes.[6] In our studies, GF109203X induces motility of PBLs, non-motile MOLT-4 cells and BW5147.0 cells as measured in a shape-change assay, whereas the classical isotype-specific inhibitor Go6976 was without effect (Fig. 3a, b). These data also support the idea that activation of a non-classical isotype maintains the non-motile state. Interestingly, neither GF109203X nor Go6976 induced shape-changing in BW5147.6 cells implying either that motility is not regulated by PKC in

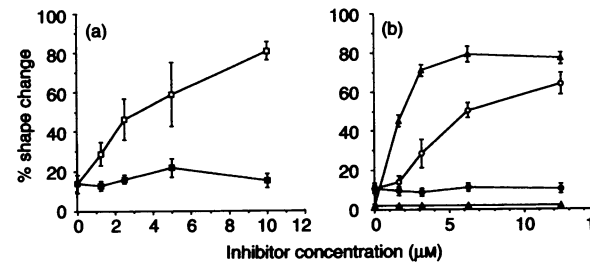


**Figure 3.** Comparison of GF109203X with Go6976 for induction of shape-change in (a) human peripheral blood T lymphocytes and (b) non-motile MOLT-4 (Δ) and BW5147.0 (○) cells. Open symbols are GF109203X and closed symbols are Go6976.

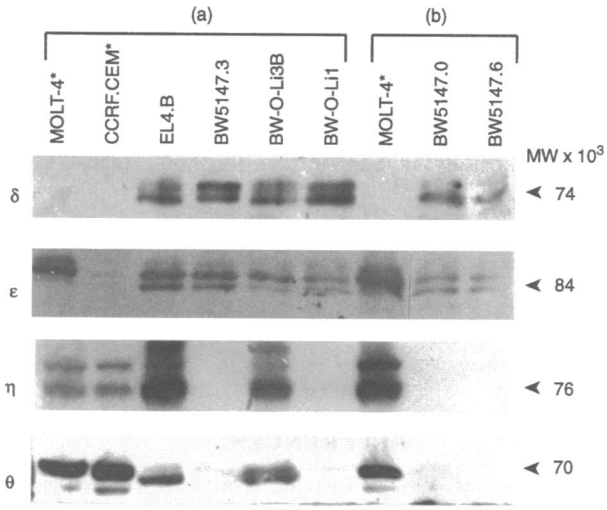


**Figure 4.** Expression of non-classical isotypes in (a) motile and (b) non-motile T cell lines as detected by Western blotting. * human cell lines, the remainder are murine.

these cells or that they lack the cellular machinery for shape-changing.

### Non-classical PKC isotype expression in motile and non-motile T lymphoid lines

Western blots of non-classical PKC-isotype expression in the motile and non-motile T-cell lines are shown in Fig. 4 and summarized in Table 1. With the exception of $\theta$ all of the non-classical PKC antibodies were rabbit polyclonals and some non-specific binding was evident. The specific protein band was therefore, confirmed by the use of appropriate blocking peptides. PKC $\zeta$ could not be detected in any of the cell lines (data not shown). The isotypes $\delta$, $\varepsilon$, $\eta$ and $\theta$ are widely expressed but there is no obvious association with motility. For example, $\varepsilon$ is present in motile MOLT-4 but not in another motile line CCRF-CEM, yet both can be rounded up by PMA (Table 2). Isotype doublets were frequently seen on the Western blots, the higher molecular weight band presumably being the phosphorylated form. However, there was no apparent difference between the ratio of the upper to lower band in motile versus non-motile cells.

### Downregulation of PKC isotypes in non-motile MOLT-4 cells and induction of motility

Previously we have shown that treatment for 44 hr with bryostatin causes downregulation of PKCs and induction of motility in non-motile MOLT-4 cells.[3] In the present study, we have compared the time courses of PKC isotype downregulation and onset of shape-change. Figure 5(a) shows that shape changing is apparent from 24 hr and increases up to 48 hr. For the classical isotypes $\alpha$ and $\beta$, downregulation is virtually complete by 24 hr but for the non-classical isotypes $\varepsilon$, $\eta$ and $\theta$ downregulation continues after 24 hr (Fig. 5b) and better correlates with the induction of shape changing.

## DISCUSSION

It is generally accepted that PKC isotype expression varies according to the cell type, differentiation status and tissue of origin. For this reason, certain cellular functions have been attributed to specific PKC isotypes and it is likely that there are still many more isotype related functions yet to be discovered. Lymphocyte motility has previously been shown to be regulated at the level of PKC.[3,5,8] These findings were largely based on agents which modify PKC activity. The role of a specific PKC isotype and its mediation of lymphocyte motility had never been addressed. We have shown in this study that

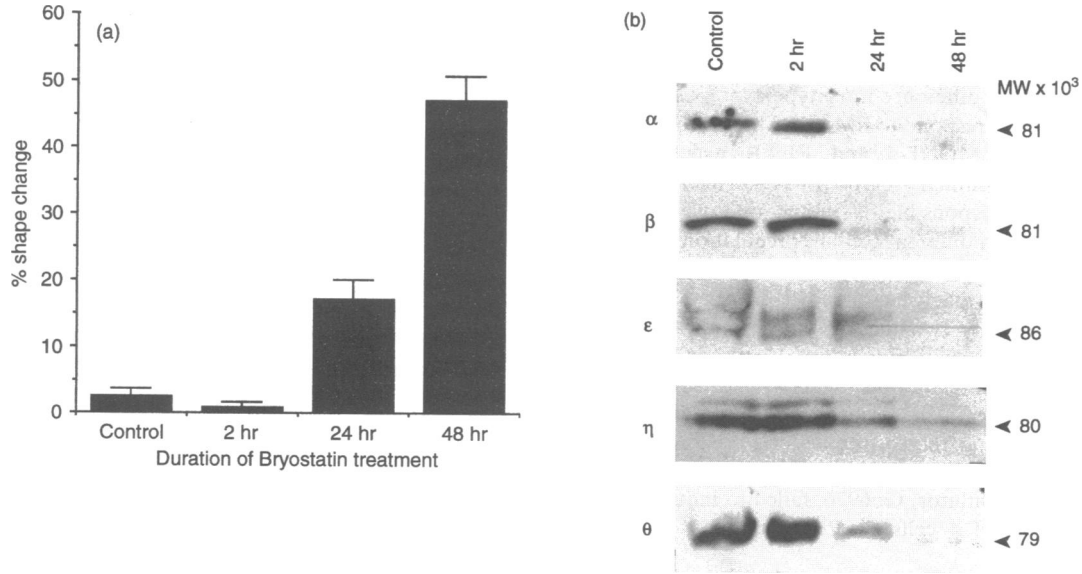


**Figure 5.** Time course for the effects of 50 nM bryostatin on (a) shape change and (b) expression of PKC isotypes $\alpha$, $\beta$, $\varepsilon$, $\eta$ and $\theta$ in non-motile MOLT-4 cells.

PKC isotype expression in the motile compared to non-motile variants did not, by itself, indicate any one of PKC isotype as a regulator of cell motility.

Exploiting our panel of T-lymphoid motility variants, PKC isotype expression was analysed at the protein level. Certain PKC isotypes were automatically eliminated as possible motility candidates. PKC $\gamma$ was not present in any of the cell lines, which was consistent with previous findings.[9] In addition, PKC $\zeta$, an atypical isotype, was also not expressed but would be an unlikely isotype, since it does not contain a PMA responsive element.[10,11] The experimental system using acute PMA treatment to induce a non-motile state, therefore, could not have been driven by PKC $\zeta$ or indeed, any other atypical isotype such as $\lambda$ and $\iota$, which is PMA unresponsive.[12,13]

PKC isotype profiles for each of the motile and non-motile cell lines varied in terms of the classical and as well as the non-classical isotypes. Whilst these differences did not correlate directly with motility it was surprising that the BW5147 variants, BW-O-Li1 and BW5147.3, did not express any of the classical PKC isotypes. Remarkably, these cells only expressed novel PKC isotypes $\delta$, $\varepsilon$ and $\theta$. The isotypes $\delta$ and $\varepsilon$ were also found in the non-motile counterparts, BW5147.0 and BW5147.6; these cells also possessed small amounts of the classical PKC isotype, $\alpha$. This was an intriguing finding and although probably unrelated to cellular shape it does imply that this rather limited array of novel PKC isotypes does not restrict growth. Indeed these cell lines which lacked the classical isotypes grew at a high rate and this is consistent with other observations that classical PKCs may be negative regulators of proliferation.[13,14]

The human MOLT-4 variants, motile and non-motile, expressed comparable levels of the same PKC isotypes. This suggests that in these cell lines PKC regulation of motility is not at the level of isotype expression, but at the level of isotype activation. This argument can also be applied to the motile line CCRF-CEM which can be rounded up by PMA, and to the non-motile line BW5147.0 which can be induced to shape change by GF109203X. In contrast, the motile lines EL4.B, BW5147.3, BW-O-Li1 and BW-O-Li3B cannot be rounded up by PMA and therefore probably do not express the isotype whose activation maintains the non-motile state. However, there is no consistent difference in isotype expression between the motile cells which respond to PMA and those which do not, and indeed motile MOLT-4 and EL4.B which respond differently have very similar isotype profiles. This implies that the PKC isotype responsible is either one we have not investigated or that in different cell lines regulation of motility is governed by different PKC isotypes.

Although our approach has failed to identify the isotype which regulates motility, two lines of evidence indicate that it is not one of the known classical isotypes. Firstly, in non-motile MOLT-4 cells, downregulation of classical isotypes with chronic bryostatin treatment occurred more rapidly than the onset of shape changing; there was a better correlation with the downregulation of non-classical isotypes. Secondly, the classical isotype specific inhibitor, Go6976, failed to induce motility in non-motile MOLT-4 cells or PBLs, unlike GF109203X which inhibits both classical and non-classical isotypes. Furthermore, we have transfected the classical isotypes $\beta$I or $\beta$II into motile BW5147.3 cells which lack PKC $\beta$ and selected $\beta$I- or II-expressing transfectants: these cells continued to shape change even after acute treatment with PMA. Therefore, on balance, this study favours a role for non-classical PKC isotypes(s) in the regulation of shape changing in lymphocytes.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Verschueren H., Van der Taelen I., Dewit J., De Braekeler J. & De Baetselier P. (1994) Metastatic competence of BW5147 T-lymphoma cell lines is correlated with *in vivo* invasiveness, motility and F-actin content. *J Leukoc Biol* **55,** 552.
2. Wilkinson P.C. & Liew F.Y. (1995) Chemoattraction of human blood T lymphocytes by interleukin-15. *J Exp Med* **181,** 1255.
3. Southern C., Wilkinson P.C., Thorp K.M., Henderson L.K., Nemec M. & Matthews N. (1995) Inhibition of protein kinase C results in a switch from a non-motile to a motile phenotype in diverse human lymphocytes populations. *Immunology* **84,** 326.
4. Stabel S. & Parker P.J. (1991) Protein kinase C. *Pharmac Ther* **51,** 71.
5. Thorp K.M., Southern C. & Matthews N. (1994) Effect of serine/threonine inhibitors on motility of human lymphocytes and U937 cells. *Immunology* **81,** 546.
6. Martiny-Baron G., Kazanietz M.G., Mischak H. *et al.* (1993) Selective inhibition of protein kinase C isoenzymes by the indolocarbazole Go6986. *J Biol Chem* **268,** 9194.
7. Laemmli E.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680.
8. Trachsel S. & Keller H.U. (1995) Selective responses (actin polymerization, shape changes, locomotion, pinocytosis) to the PKC inhibitor RO 31-8220 suggest that PKC discriminately regulates functions of human blood lymphocytes. *J Leukoc Biol* **57,** 587.
9. Lucas S., Marais R., Graves J.D., Alexander D., Parker P. & Cantrell D.A. (1990) Heterogeneity of protein kinase C expression and regulation in T lymphocytes. *FEBS Lett* **260,** 53.
10. McGlynn E., Liebetanz J., Reutener S. *et al.* (1992) Expression and partial characterisation of rat protein kinase C-delta and protein kinase C-zeta in insect cells using recombinant baculovirus. *J Cell Biochem* **49,** 239.
11. Ono Y., Fujii T., Ogita K., Kikkawa U., Igarashi K. & Nishizuka Y. (1989) Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties. *Proc Natl Acad Sci* **86,** 3099.
12. Hug H. & Sarre T.F. (1993) Protein kinase enzymes: divergence in signal transduction? *Biochem J* **291,** 329.
13. Dekker L.V. & Parker P.J. (1994) Protein kinase C—a question of specificity. *Trends Biochem Sci* **19,** 73.
14. Gamard C.J., Blobe G.C., Hannun Y.A. & Obeid L.M. (1994) Specific role for protein kinase C beta in cell differentiation. *Cell Growth Differ* **5,** 405.
15. Cuadrado A., Issing W., Fleming T.P. & Molloy C.J. (1994) Uneven distribution of protein kinase C alpha and beta isozymes in human sarcoma and carcinomas. *J Cell Physiol* **159,** 434.