The effects of phorbol ester, diacylglycerol, phospholipase C and Ca^{2+} ionophore on protein phosphorylation in human and sheep erythrocytes

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1. Treatment of human or sheep erythrocytes with PMA (phorbol myristate acetate) enhanced [^{32}P]phosphate labelling of membrane polypeptides of approx. 100, 80 and 46 kDa. The 80 kDa and 46 kDa polypeptides coincided with bands 4.1 and 4.9 respectively on Coomassie-Blue-stained gels. 2. Similar but smaller effects were obtained by treating human cells with 1-oleoyl-2-acetyl-*rac*-glycerol (OAG), exogenous bacterial phospholipase C or ionophore A23187 + Ca^{2+}, each of which treatments would be expected to raise the concentration of membrane diacylglycerol. In contrast, sheep cells, which do not increase their content of diacylglycerol when treated with phospholipase C or A23187 + Ca^{2+}, only showed enhanced phosphorylation with OAG. Neither human nor sheep cells showed any enhanced [^{32}P]phosphate labelling of phosphoproteins when treated with 1-mono-oleoyl-*rac*-glycerol. 3. It is concluded that diacylglycerol from a variety of sources can activate erythrocyte protein kinase C, but that the most effective diacylglycerol is that derived from endogenous polyphosphoinositides. 4. In contrast with bacterial phospholipase C and A23187, which stimulate synthesis of phosphatidate by increasing the cell-membrane content of diacylglycerol in human erythrocytes, PMA, OAG or 1-mono-oleoyl-*rac*-glycerol caused no change in phospholipid metabolism.

INTRODUCTION

Progress in our understanding of the mechanisms through which cell-surface receptor activation leads to intracellular responses has advanced rapidly, particularly in those cases where cyclic AMP appears to be a second messenger (Gilman, 1984). However, in the many cellular situations where receptor activation does not involve activation of adenylate cyclase, it is only recently that the mechanism of message transmission has become clearer. Present evidence supports a scheme whereby receptor-activated breakdown of inositol phospholipids and consequent mobilization of Ca^{2+} and activation of a Ca^{2+}- and phospholipid-sensitive protein kinase (C) play a crucial role in stimulus–response coupling (Berridge, 1984; Nishizuka, 1984).

Perhaps the most novel aspects of this scheme are the proposals that diacylglycerol derived by phosphodiesterase cleavage of triphosphoinositide can directly activate protein kinase C and, moreover, that PMA, a potent cell activator and co-carcinogen, functions as a non-metabolizable analogue of diacylglycerol, binding to protein kinase C and promoting the phosphorylation of certain membrane proteins in the same way as the natural activator. Protein kinase C activity seems to be ubiquitous in mammalian cells and is even present in erythrocytes, as judged by the enhanced phosphorylation of membrane proteins after treatment of rabbit erythrocytes with PMA (Ling & Sapirstein, 1984). Of particular interest is the marked increase in phosphorylation of band 4.1, a polypeptide which appears to have a crucial role in mediating the interaction of spectrin and actin in the erythrocyte membrane skeleton (Goodman & Shiffer, 1983).

In the work presented here we have confirmed the work of Ling & Sapirstein (1984) and in addition have demonstrated that diacylglycerol added exogenously (as OAG) or endogenously (by phospholipase C action) gives a pattern of phosphoprotein labelling which is very similar to that given by PMA. Most significantly, we have also shown that diacylglycerol derived from polyphosphoinositides in human (but not sheep) cells is a potent activator of protein kinase C.

MATERIALS AND METHODS

Materials

[^{32}P]Phosphate (carrier free, 10 mCi/ml) was obtained from Amersham International p.l.c. (Amersham, Bucks., U.K.). *Clostridium welchii* phospholipase C was supplied by Wellcome Research (Beckenham, Kent, U.K.). All the other reagents were from either Sigma Chemical Co. (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

Preparation and incubation of cells

Blood was obtained either from sheep, with EGTA as anticoagulant, or from various human donors, with heparin as anticoagulant. Fresh blood samples were centrifuged at 1000 *g* for 5 min, and erythrocytes were washed three times with 150 mM-NaCl, with removal of the buffy coat. The cells were then given a final wash with 20 mM-Mops/NaOH buffer, pH 7.1, containing 150 mM-NaCl (Mops/saline). Washed erythrocytes were

Abbreviations used: PMA, phorbol myristate acetate; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; SDS, sodium dodecyl sulphate.

resuspended at approx. 25% haematocrit in Mops/saline containing 10 mM-glucose, 1 mM-Mg^{2+} and 400 μCi of [^{32}P]phosphate/ml of packed cells and incubated at 37 °C for 90 min. After 90 min, 0.2 ml samples were added to 0.8 ml of Mops/saline and were incubated for a further 60 min in the presence of various concentrations of PMA, OAG, 1-mono-oleoyl-*rac*-glycerol or ceramide. PMA was added in dimethylsulphoxide solution, and OAG, 1-mono-oleoyl-*rac*-glycerol and ceramide were added as sonicated suspensions in Mops/saline. Similar samples of [^{32}P]phosphate-labelled erythrocytes in Mops/saline containing 1 mM-Ca^{2+} were treated with either up to 2 μg of *Cl. welchii* phospholipase C or up to 5 μM ionophore A23187 in a total volume of 1 ml and incubated at 37 °C for a further 20 min. Incubations were stopped by addition of 50 μl of 100 mM-EGTA.

Analysis of membrane polypeptides by SDS/polyacrylamide-gel electrophoresis

Membranes were prepared from erythrocytes by lysis at 4 °C in 10 vol. of 10 mM-Hepes/NaOH buffer, pH 7.5, containing 1 mM-Mg^{2+} and 0.1 mM-EGTA, followed by three washes in the same medium. Analysis of membrane polypeptide patterns was carried out by SDS polyacrylamide-gel electrophoresis on 10% (w/w)-acrylamide gels, by the procedure of Laemmli (1970). Rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), bovine muscle actin (43 kDa) and soya-bean trypsin inhibitor (20.5 kDa) were used as standard M_r markers. Gels were dried at 80 °C under vacuum after staining with Coomassie Brilliant Blue R (0.05%, w/w). Dried gels were autoradiographed by exposure to Kodak XRP X-ray film for 3–5 days.

Polypeptides were quantified, after staining the gels with Coomassie Brilliant Blue R, by scanning with a Bio-Rad scanning densitometer (model 1650) linked to a Shimadzu C-RIB integrator. [^{32}P]Phosphate incorporation into various phosphoproteins, and total phospholipid was quantified either by scanning the autoradiograms or by excising various polypeptide or lipid bands from either wet or dried gels and counting the Čerenkov radiation in 10 ml of water.

Measurement of ATP specific radioactivity

Trichloroacetic acid (final concn. 5%, w/v) was added to samples of erythrocytes and, after centrifugation at 12000 *g* for 2 min at 4 °C, the clear supernatant solution was extracted with 4 × 10 vol. of diethyl ether to remove trichloroacetic acid. Samples of the solution were analysed by h.p.l.c. by the procedure of Anderson & Murphy (1976). The ATP peak was quantified chemically by using a Waters Lambda Max spectrophotometer set at 260 nm in conjunction with a Shimadzu C-RIB integrator. ATP radioactivity was counted by its Čerenkov radiation in 10 ml of water.

Separation and quantification of phospholipids

These procedures were carried out as described by Allan *et al.* (1980). Polyphosphophoinositides were separated by t.l.c. on 1%-oxalate-impregnated silica-gel H plates with a solvent consisting of chloroform/methanol/acetone/acetic acid/water (40:13:15:12:7, by vol.).

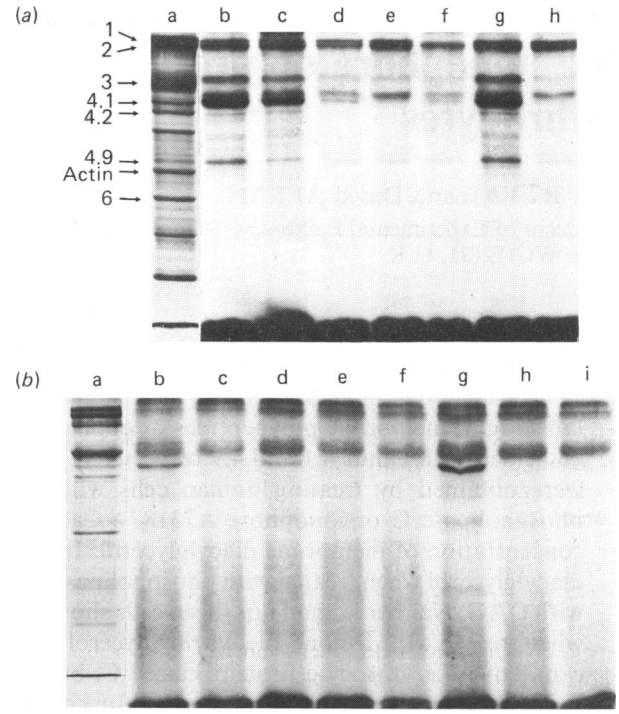

Fig. 1. SDS/polyacrylamide-gel electrophoresis of membrane polypeptides from human (*a*) and sheep (*b*) erythrocytes treated with PMA, phospholipase C, OAG, 1-mono-oleoyl-*rac*-glycerol, ceramide or A23187/Ca^{2+}

[^{32}P]Phosphate-labelled human erythrocytes (*a*) (~ 5% haematocrit in Mops/saline) were incubated at 37 °C with PMA (100 nM or 1 μM, 60 min) (b, g), phospholipase C (2 μg, 20 min) (c), OAG (200 μM, 60 min) (d), 1-mono-oleoyl-*rac*-glycerol (200 μM, 60 min) (e), A23187 + Ca^{2+} (5 μM, 20 min) (f), or no addition (20 min) (a, h). Membrane fractions were prepared and electrophoresed on 10% (w/w) gels as described in the Materials and methods section. Lane (a) represents a Coomassie-Blue-stained control membrane polypeptide pattern; lanes (b)–(h) represent autoradiograms of [^{32}P]phosphate-labelled phosphoproteins derived from various treated samples. [^{32}P]Phosphate-labelled sheep erythrocytes (*b*) were incubated with various additions as described for human cells, with the following exceptions. Cells were incubated at 37 °C with PMA (100 nM) for either 20 min (b) or 60 min (g), or with ceramide (200 μM, 60 min) (i). Lane (a) represents a Coomassie-Blue-stained control membrane polypeptide pattern. Lanes (b)–(i) represent autoradiograms of [^{32}P]phosphate-labelled phosphoproteins derived from various treated samples. Nomenclature of polypeptide bands is as described by Steck (1974). In some experiments, notably with sheep erythrocytes treated with PMA, a small increase (< 20%) was observed in [^{32}P]phosphate labelling of the polyphosphoinositides. Further investigation suggested that this was due to contaminating buffy-coat cells. The ability of PMA to increase polyphosphoinositide labelling has been reported for neutrophils (Cockcroft *et al.*, 1985), platelets (de Chaffoy de Courcelles *et al.*, 1984*b*) and lymphocytes (Taylor *et al.*, 1984).

RESULTS

Fig. 1 shows polypeptide patterns and corresponding autoradiograms of membranes from human and sheep erythrocytes which had been labelled with [^{32}P]phosphate and then treated with PMA, OAG, 1-mono-oleoyl-*rac*-

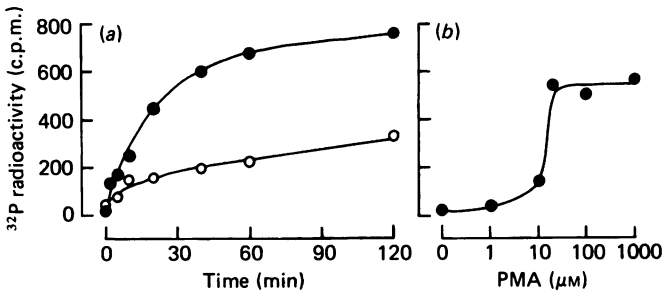

Fig. 2. Time course (*a*) and dose–response curve (*b*) of [^{32}P]phosphate labelling of human erythrocyte membrane proteins in cells treated with PMA

Samples of [^{32}P]phosphate-labelled erythrocytes were resuspended at ~ 5% haematocrit either in (*a*) Mops/saline containing 100 nM-PMA and incubated at 37 °C for various times or in (*b*) Mops/saline containing various concentrations of PMA (0–1000 nM) and incubated at 37 °C for 60 min. Plasma membranes were prepared and [^{32}P]phosphate-labelled phosphoproteins, including band 4.1 (●) and 100 kDa band (○), were quantified as described in the Materials and methods section. The results are shown from a single experiment in which each point represents the mean of duplicate determinations. Two similar experiments using different batches of cells gave essentially the same results. The time course and dose–response curve for [^{32}P]phosphate-labelled phosphoproteins from sheep erythrocytes were similar to the results shown here.

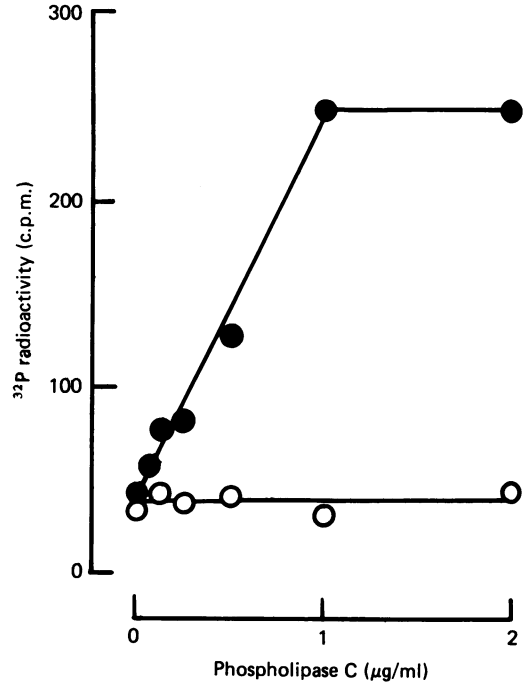

Fig. 3. Effect of phospholipase C concentration on [^{32}P]phosphate labelling of band 4.1 and spectrin in human erythrocytes

[^{32}P]Phosphate-labelled erythrocytes were incubated at ~ 5% haematocrit in Mops/saline containing 1 mM-Ca^{2+} with various concentrations of *Cl. welchii* phospholipase C (0–2 μg/ml) at 37 °C for 20 min. Enzyme action was stopped by addition of 50 μl of 100 mM-EGTA. [^{32}P]Phosphate labelling of band 4.1 (●) and spectrin (○) was analysed as described in the Materials and methods section. Each point represents the mean of duplicate determinations and similar results were observed in three further experiments with different batches of cells. In the experiment shown here, 270 c.p.m. was measured in band 4.1 in a sample treated with 0.1 μM-PMA for 20 min.

glycerol, ceramide, phospholipase C or A23187. Essentially, the Coomassie-Blue-stained polypeptide pattern of both the human and sheep erythrocyte membranes is similar, the main difference being that sheep, but not human, membranes possess a polypeptide band of approx. 180 kDa. Likewise, the autoradiograms show a similar pattern of [^{32}P]phosphate labelling of phosphoproteins in the membranes from the two different sources, the major difference being the relatively small labelling of spectrin in sheep erythrocytes. PMA enhances the [^{32}P]phosphate labelling of several human or sheep erythrocyte membrane polypeptides, particularly those of 100, 80 and 46 kDa. The 80 kDa radioactive polypeptide coincides with band 4.1 on Coomassie-Blue-stained patterns, as shown by Ling & Sapirstein (1984), using rabbit cells, and the 46 kDa radioactive polypeptide coincides with band 4.9. The optimal concentration of PMA was about 100 nM, and labelling was maximal after about 60 min (Fig. 2). From the radioactivity in band 4.1 and from the measured ATP specific radioactivity it was calculated that band 4.1 can incorporate about 3–4 mol of phosphorus/mol of protein during treatment with PMA. No obvious changes in cell morphology were apparent in cells treated with PMA.

Treatment with *Cl. welchii* phospholipase C caused a very similar labelling of the same polypeptides that were labelled with PMA (Figs. 1 and 3). Maximal incorporation into band 4.1 was $90 \pm 20\%$ (S.D.; $n = 4$) of that obtained with PMA and occurred with 1 μg of phospholipase C/ml, where total diacylglycerol formed was about 10 nmol/10^9 cells. This value was calculated by measuring the breakdown of phosphatidylcholine in the cells and subtracting the amount of phosphatidate synthesized as a result of diacylglycerol kinase activity (Allan *et al.*, 1975). Labelling of band 4.1, synthesis of phosphatidate and generation of diacylglycerol increased linearly with phospholipase C concentration up to 1 μg/ml. No enhancement of phosphoprotein labelling was obtained on treating sheep erythrocytes with phospholipase C, evidently because there are no diacylglycerolipids on the external lipid leaflet of the sheep cell membranes (Nelson, 1972). Ceramide, the sphingolipid analogue of diacylglycerol, was formed in large quantities, but apparently had no effect on protein labelling (Fig. 1*b*).

Addition of OAG (200 μM) to either human or sheep cells caused an increase in phosphoprotein labelling equivalent to about 20–30% of that obtained with PMA (Figs. 1 and 4). However, no increase was observed in the labelling of phosphatidate in either human or sheep cells, suggesting that OAG is not phosphorylated in measurable amounts. Treatment of human or sheep erythrocytes with an identical concentration of 1-mono-oleoyl-*rac*-glycerol induced no enhanced labelling of phosphoproteins or phospholipids in either system.

An increase of Ca^{2+} within human (but not sheep) erythrocytes is known to generate diacylglycerol by activation of a polyphosphoinositide phosphodiesterase (Allan & Michell, 1977, 1978), and we considered the possibility that this diacylglycerol can also activate

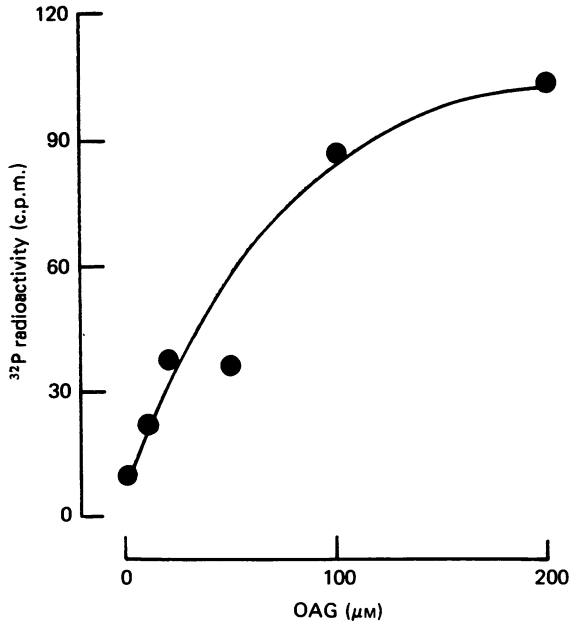

Fig. 4. Dose–response curve of [^{32}P]phosphate labelling of band 4.1 in human erythrocytes treated with OAG

[^{32}P]Phosphate-labelled human erythrocytes were incubated at ~ 5% haematocrit in Mops/saline for 60 min at 37 °C in the presence of various concentrations of OAG (0–200 μM). After 60 min, the cells were lysed and [^{32}P]phosphate labelling of band 4.1 was analysed as described in the Materials and methods section. Each point represents the mean of duplicate determinations, and similar results were observed in three further experiments using different batches of erythrocytes. In the experiment shown here, a sample treated for 60 min with 0.1 μM-PMA gave 600 c.p.m. in band 4.1.

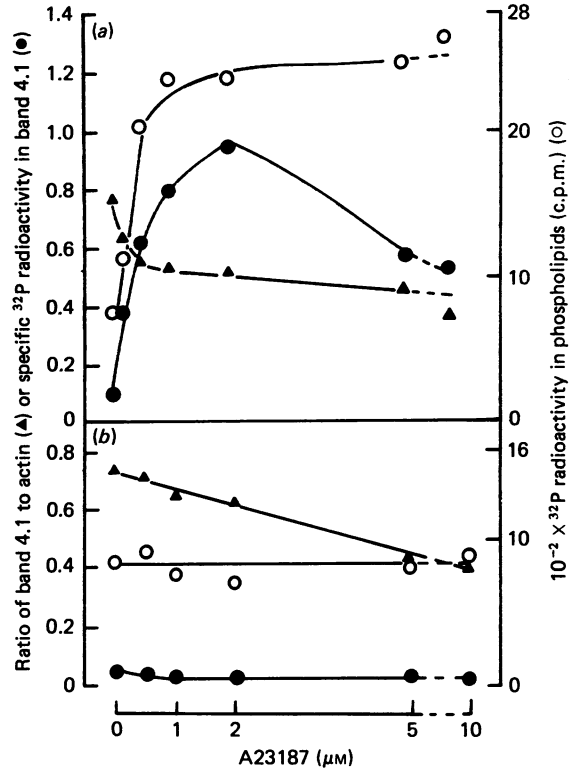

Fig. 5. Effect of A23187 concentration on the [^{32}P]phosphate labelling of band 4.1 and phospholipids in (*a*) human and (*b*) sheep erythrocytes

[^{32}P]Phosphate labelled cells were incubated at 37 °C for 20 min at ~ 5% haematocrit in Mops/saline containing 1 mM-Ca^{2+} and various concentrations of A23187 (0–10 μM). Incubations were stopped by addition of 100 mM-EGTA to give a final concentration of 5 mM. Plasma membranes were prepared, and the [^{32}P]phosphate labelling of band 4.1 and phospholipids was analysed as described in the Materials and methods section. The results shown are from a single experiment in which each point represents the mean of duplicate determinations. Two further experiments using different batches of cells gave essentially similar results. ▲, Ratio of band 4.1:actin as measured by densitometry of a Coomassie-Blue-stained gel. ●, Specific ^{32}P radioactivity of band 4.1 expressed as a ratio of c.p.m.:Coomassie-Blue-stain intensity of the same band (arbitrary units). In the experiment shown here, samples treated with 0.1 μM-PMA for 20 min gave values of 3.68 (human) and 8.57 (sheep) units. ○, ^{32}P radioactivity (c.p.m.) in total phospholipids at the gel front. Subsidiary experiments using t.l.c. separation of phospholipids showed that all the increase in radioactivity produced by A23187/Ca^{2+} treatment was due to an increment in the radioactivity of phosphatidate (see also Allan *et al.*, 1976).

protein kinase C. We therefore examined the effect of ionophore A23187 + Ca^{2+} on phosphoprotein labelling. Addition of A23187 + Ca^{2+} to either sheep or human cells caused a loss of band 4.1, presumably as a result of proteolytic activity (Figs. 1 and 5) as described previously (Allan & Thomas, 1981). However, in human, but not in sheep, cells there was a marked increase in phosphorylation of band 4.1 under the same conditions, corresponding to about 25–35% of the effect of PMA. The contrast between the behaviour of the two cell types is probably related to the fact that sheep cells do not possess the Ca^{2+}-sensitive polyphosphoinositide phosphodiesterase present in human erythrocyte membranes and thus have no mechanism for generating endogenous diacylglycerol (Allan & Michell, 1977).

DISCUSSION

The experiments described here generally support the view (Berridge, 1984; Nishizuka, 1984) that PMA and diacylglycerol have a common action in stimulating protein kinase C, since they give rise to very similar (although quantitatively different) patterns of [^{32}P]phosphate labelling of erythrocyte membrane protein. Of the various agents used, OAG (Fig. 4) seemed to be relatively the least effective, probably because the hydrophobic properties of this compound make it difficult to deliver it effectively into the erythrocyte membrane, and this may also be the reason why phosphorylation of OAG to form phosphatidate was not observed. It is noteworthy that phosphorylation of OAG was seen in platelets (Kaibuchi *et al.*, 1983; de Chaffoy de Courcelles *et al.*, 1984*a*). Generation of diacylglycerol directly within the membrane by treatment of human cells with exogenous bacterial phospholipase C caused a large increase in phosphatidate formation (Allan *et al.*, 1975) and in protein phosphorylation (Figs. 1 and 3). *Cl. welchii* phospholipase C mainly attacks phosphatidylcholine and

sphingomyelin in intact cells, so that the diacylglycerol formed would have the fatty acid composition characteristic of phosphatidylcholine and would need to cross the membrane bilayer in order to activate protein kinase C. Present evidence suggests that passage of diacylglycerol across the membrane is rapid, with $t_{\frac{1}{2}} \sim 15$ s (Ganong & Bell, 1984). Thus, in the time scale of these experiments, diacylglycerol produced at the outer surface would very quickly gain access to the inner lipid leaflet. On a weight-for-weight basis, diacylglycerol introduced into the membrane by phospholipase C treatment seemed to be about 100 times as effective as OAG. Ceramide, which is formed from sphingomyelin by phospholipase C and which bears some structural similarity to diacylglycerol, did not activate protein kinase C in sheep cells.

Introduction of Ca^{2+} into human erythrocytes is an effective procedure for activating protein kinase C (Figs. 1 and 5), but, because of the complex series of biochemical changes caused by Ca^{2+} (Allan & Thomas, 1981), it is not easy to determine the mechanism of the protein kinase activation. It is possible that Ca^{2+} directly activates the kinase (Nishizuka, 1984), but this explanation does not seem to apply to sheep erythrocytes, since no enhanced protein phosphorylation was observed in these cells treated with A23187 + Ca^{2+}, although they possess protein kinase C, as judged by their response to PMA and diacylglycerol (Fig. 1*b*). It has been suggested that protein kinase C could be activated by a Ca^{2+}-controlled thiol proteinase (Kikkawa *et al.*, 1982), and indeed such proteinases are present in erythrocytes of different species (Allan & Thomas, 1981; Thomas *et al.*, 1983). Again, however, no activation of protein kinase C was seen in sheep cells under conditions where Ca^{2+} was clearly activating a proteinase, as judged by the progressive disappearance of band 4.1 (Fig. 5).

In human, but not sheep, erythrocytes, Ca^{2+} entry promotes the breakdown of polyphosphoinositides by a phosphodiesterase to give diacylglycerol, which is then largely converted into phosphatidate (Allan *et al.*, 1976). There is no evidence that protein kinase C can be activated by phosphatidate, and we are left with the suggestion that the small amounts of diacylglycerol which accumulate during treatment of human erythrocytes with A23187 are sufficient to cause protein kinase C activation. A previous estimate (Allan *et al.*, 1976) suggests that only about 1 nmol of diacylglycerol is formed in 10^9 cells treated for 15 min with A23187, so that this endogenous form of diacylglycerol is approx. 200-fold as effective as exogenous OAG and 3-fold as effective as bacterial phospholipase C-derived diacylglycerol. Possibly the characteristic stearoyl–arachidonoyl fatty acid composition of diacylglycerol derived from polyphosphoinositides contributes to its relative effectiveness.

Although there is now good evidence that diacylglycerol is the natural activator of protein kinase C (Nishizuka, 1984), it has not convincingly been shown that diacylglycerol derived from endogenous polyphosphoinositides can directly activate protein kinase C in cells. Our present data go some way to providing this direct evidence by demonstrating that protein kinase C is activated under conditions where diacylglycerol increases at the expense of polyphosphoinositides.

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