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## Human neutrophil protein kinase C: calcium-induced changes in the solubility of the enzyme do not always correlate with enzymatic activity

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We hypothesized that calcium and 1,2-diacylglycerols stimulated human neutrophil (PMN) protein kinase C (EC 2.7.1.37) in a two-step mechanism. The proposed mechanism entails (1) increased insoluble protein kinase C activity and (2) endogenous protein phosphorylation, events which have not been biochemically dissociated. PMN which were treated with 100 nM ionomycin shifted protein kinase C activity from being mostly soluble to insoluble. Concentrations of ionomycin greater than 300 nM stimulated a doubling of total cellular (soluble + insoluble) protein kinase activity and stimulated increased endogenous phosphorylation of PMN proteins. Intracellular calcium (measured with fura-2) increased from 65 nM (basal) to 680 nM using 500 nM ionomycin; calcium increases were dose-dependent. The anti-inflammatory agents acetylsalicylic acid and sodium salicylate (but not ibuprofen, indomethacin or acetaminophen) inhibited ionomycin-induced protein kinase C activation and protein phosphorylation in a dose-dependent manner by inhibiting the production of diacylglycerols. 1-Oleoyl-2-acetyl-glycerol reversed the inhibitory effect of salicylates. In contrast to the effect of acetylsalicylates on protein kinase C functional activity the distribution of phorbol receptors was unaffected in acetylsalicylate-treated, ionomycin-stimulated PMN using a phorbol-binding assay. Our results show that ionomycin increased intracellular diacylglycerol levels 3.5-fold over those present in control PMN, while acetylsalicylate decreased diacylglycerol production in ionomycin-stimulated PMN below baseline values. These results support the hypothesis that increased intracellular calcium activated protein kinase C leading to protein phosphorylation in two distinct dissociable events: (1) increased intracellular calcium; and (2) increased 1,2-diacylglycerol levels.

Abbreviations:  $[Ca^{2+}]_i$ , intracellular calcium concentration; DMSO, dimethyl sulfoxide; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride; fura-2/AM, fura-2 acetoxymethyl ester; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PDB, phorbol 12,13-dibutyrate;  $[^3H]PDB$ ,  $[20(n)-^3H]$ phorbol 12,13-dibutyrate; PMN, polymorphonuclear leukocytes; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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### Introduction

Certain surface receptors, when bound by their specific ligands, activate guanine nucleotide-binding proteins (G-proteins) [1,2]. G-proteins in turn are known to activate phospholipase C, which converts phosphatidylinositol 1,4-bisphosphate to *myo*-inositol 1,4,5-trisphosphate and diacylglycerol (reviewed in Ref. 3). Inositol trisphosphate induces a rise in the concentration of intracellular calcium ( $[Ca^{2+}]_i$ ) which is released mostly from

intracellular pools. Calcium and phospholipid-dependent protein kinase has been described in many tissues [4-6], and is found in both soluble (over 75% of the total cellular activity) and insoluble forms. Studies in rabbit polymorphonuclear leukocytes (PMN) suggest that at least membrane-bound protein kinase C is regulated through a guanine nucleotide-dependent pathway [6]. One proposed protein kinase C activation mechanism states that calcium and 1,2-diacylglycerols provide dual signals [7,8] that stimulate protein kinase C to alter its solubility properties from mostly soluble to insoluble [4,5,9]. The change in protein kinase C solubility may be associated with its binding to four or more phospholipid residues (mostly phosphatidyserine) which are associated with cellular membranes [10]. It is unclear whether protein kinase C physically moves from the cytosol to membranes, or if protein kinase C 'translocation' merely reflects changes in enzyme activity which are associated with specific cellular microenvironments. The existence of a calcium/phospholipid-independent form of protein kinase C [11,12] and knowledge of genetically different subsets of protein kinase C [13,14] demand that current models of protein kinase C activation be altered in order to accommodate the regulation of the different types of protein kinase C, a multifunctional enzyme with at least 35 documented substrates. Increased affinity of activated protein kinase C for cellular membranes may function to bring protein kinase C into close proximity with reported substrates, such as the receptors for epidermal growth factor insulin, and transferrin, the phosphorylation of which is correlated with the regulation of receptor endocytosis and selective receptor down-regulation [15-17]. With regard to the regulation of cellular activation, other enzymes are reported to phosphorylate proteins which are also substrates of protein kinase C. For example, protein kinase C and myosin light chain kinase both phosphorylate the 20 kDa light chain of myosin [18,19] but at different amino-acid residues. In summary, these reports suggest the extreme complexity of interpreting results from studies attempting to define a role for protein phosphorylation by protein kinase C during PMN activation.

We now report that protein kinase C activation

involves at least two steps which can be functionally separated into changes in the solubility of the enzyme's activity and changes in protein phosphorylation. Our results indicate that increased  $[Ca^{2+}]_i$  alone, if sufficiently high in concentration, can stimulate increased insoluble protein kinase C activity, but this observation does not always indicate that significant qualitative or quantitative changes in cellular phosphoproteins have occurred. A second stimulus involving diacylglycerol production is apparently required to activate membrane associated-protein kinase C to phosphorylate proteins within the cell.

## Materials and Methods

**Cells.** Human PMN were obtained from freshly drawn, whole blood by venipuncture of normal, healthy adult donors. The PMN were isolated by Ficoll/hypaque centrifugation, dextran sedimentation, and hypotonic lysis of residual erythrocytes as described [20,21]. Purified PMN were washed with 100 mM phosphate-buffered saline or 10 mM Hepes (pH 7.5) containing 10 mM magnesium and 0.5 mM calcium before use. Cells were greater than 97% PMN by differential staining, and were greater than 95% viable by the Trypan blue exclusion assay.

**Materials.** Ionomycin was obtained from Calbiochem (La Jolla, CA) and stored at 4°C as a 10 mg/ml (14 mM) stock solution in DMSO. H-7 was obtained from Seikagaku America (St. Petersburg, FL) and was diluted with the appropriate buffer from a 10 mM aqueous stock solution. The acetoxymethyl ester of fura-2 was purchased from Molecular Probes (Junction City, OR) and stored at -20°C as a 1 mg/ml stock solution. Radio-labeled compounds were obtained from Amersham (Arlington Heights, IL). Reagents used in electrophoresis were from Bio-Rad (Rockville Center, NY). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated. Protein concentrations were determined using the Pierce reagent (Pierce, Rockford, IL).

**Intracellular calcium measurements using fura-2.** For these experiments, cells were isolated from whole blood using citric acid/dextrose. A 30 ml aliquot of fresh, non-heparinized blood was mixed with 6 ml of ACD (220 mM dextrose/200 mM

trisodium citrate/140 mM citric acid), plus 18 ml of 6% dextran/0.5% NaCl (Abbott Laboratories, Chicago, IL) and sedimented for 45 min at room temperature. PMN-rich supernatant was centrifuged at  $500 \times g$  for 6 min, and the resulting pellet was subjected to two rounds of hypotonic shock to lyse erythrocytes. The cellular pellet was mixed with 2 ml of 100 mM phosphate-buffered saline, layered onto 3 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at  $850 \times g$  for 20 min at  $4^\circ\text{C}$ . The final pellet contained more than 97% neutrophils and was used for the experiments described below.

PMN at  $5 \cdot 10^7/\text{ml}$  were incubated for 25 min at room temperature in 10 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  plus 5  $\mu\text{M}$  fura-2/AM. Loaded cells were washed twice to remove unincorporated fura-2, and pre-warmed for 10 min at  $37^\circ\text{C}$ . Some cells were pre-warmed in calcium- and magnesium-free loading buffer containing 2 mM EDTA. Intracellular calcium measurements were made using a Perkin-Elmer model 650-10S fluorescence spectrophotometer and a continuously stirred cuvette. The reaction mixture contained  $10^6$  cells in 1 ml of loading buffer in the presence or absence of stimuli or inhibitors. Fura-2 fluorescence measurements were made at 500 nm emission and at both 340 nm and 380 nm excitation wavelengths [22]. Maximal fluorescence was obtained by permeabilizing the cells with 15  $\mu\text{g}/\text{ml}$  digitonin, and minimal fluorescence was obtained by competing calcium from fura-2 with 2.5 mM  $\text{MnCl}_2$ . Calcium concentrations were derived from direct measurements of the spectrophotometric tracings using the following formula:

$$[\text{Ca}^{2+}] = \left( \frac{R - R_{\min}}{R_{\max} - R} \right) K_d \cdot B$$

where  $R$  is the ratio of the fluorescence excitation measurements at 340 to 380 nm,  $R_{\min}$  and  $R_{\max}$  are the experimentally determined minimum and maximum calcium measurement ratios (340 nm to 380 nm), respectively,  $K_d$  is the dissociation constant for fura-2, and  $B$  is a fixed dye calibration constant.

*Cell treatment with ionomycin and salicylates.* PMN were pre-warmed for 5 min at  $5 \cdot 10^6$

cells/ml in either 100 mM phosphate-buffered saline or 10 mM Hepes (pH 7.4) plus 10 mM  $\text{Mg}^{2+}$  and 500  $\mu\text{M}$   $\text{Ca}^{2+}$ . PMN were centrifuged and resuspended in pre-warmed buffer containing the appropriate concentration of ionomycin for 5 min. At the end of the incubation period, 10 vol. of ice-cold buffer was added to the reaction vessel and the cells were immediately centrifuged. For some experiments, PMN were first treated for 5 min with 0.001–100  $\mu\text{M}$  concentrations of anti-inflammatory agents before treatment with ionomycin; anti-inflammatory agents were present throughout the incubation period.

*Cell fractionation and protein kinase C assay.* PMN were disrupted by sonication in homogenization buffer (50 mM Tris (pH 7.5), 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF and 0.05% Triton X-100) and centrifuged at  $100000 \times g$  for 60 min to prepare soluble and insoluble fractions, as previously described [23]. Aliquots of fractions were assayed for protein kinase C activity in vitro within 24 h of their preparation, as described [23,24], using histone type IIIS as a substrate. Assays were conducted to quantitate background phosphotransferase activity in the assay by measuring histone phosphorylation in the absence of calcium, activators and phospholipid; under these conditions, background activity was routinely less than 10% of the total protein kinase C activity; total cellular protein kinase C activity was defined as the sum of the soluble and insoluble activities recovered from a single population of PMN treated as indicated. Negative control conditions consisted of assays at 4 or  $40^\circ\text{C}$ , in the absence of sample (i.e., protein kinase C-containing material), or using sample which had been boiled for 3 min before adding them to the assay. Positive control conditions included parallel assays using highly purified protein kinase C, or protein kinase C activity stimulated by 10 ng/ml phorbol 12-myristate 13-acetate, a potent protein kinase C activator. Protein kinase C activity was calculated by subtracting both negative control and background activities, and is reported as pmol [ $^{32}\text{P}$ ]phosphate transferred to histone protein per min for  $10^{-7}$  cells,  $\pm$ S.E.

*[ $^3\text{H}$ ]Phorbol 12,13-dibutyrate-binding assays.* Cellular phorbol ester receptors were quantitated in soluble and insoluble neutrophil fractions

[25,26]. To accomplish this, 100  $\mu$ l of subcellular fraction (adjusted to 200  $\mu$ g/ml protein) were added to a mixture of 50 mM Tris-HCl (pH 7.5) 100  $\mu$ g/ml phosphatidylserine, 1 mM  $\text{CaCl}_2$ , 2 mg/ml bovine serum albumin and 100 nM [ $^3\text{H}$ ]PDB (19.7 Ci/mmol, 39 mCi/mg). Binding proceeded for 30 min at 37°C with constant shaking; the reaction was stopped by adding 1 ml of 20% trichloroacetic acid. Bound [ $^3\text{H}$ ]PDB was separated from free [ $^3\text{H}$ ]PDB by collecting the trichloroacetic acid precipitates by filtration onto 0.45  $\mu$ m type HA filter membranes (Millipore); filters were washed twice with 10% trichloroacetic acid and dried, and the precipitated radioisotope was quantitated by liquid scintillation counting in 10 ml of Hydrofluor (National Diagnostics). Non-specific binding was determined in the presence of 100  $\mu$ M unlabeled PDB.

*PMN loading with [ $^{32}\text{P}$ ]phosphate.* PMN were loaded with [ $^{32}\text{P}$ ]phosphate by modifying previously published methods [23,24,27] so as to maximize [ $^{32}\text{P}$ ]phosphate loading and minimize both cellular activation which was caused by PMN adherence and background phosphorylation in resting PMN. PMN ( $10^8$  cells/condition) were incubated for 30 min at 25°C in 100 mM phosphate-buffered saline containing 450  $\mu$ M calcium and 250  $\mu$ M magnesium for 30 min at 25°C. The medium was replaced with incubation buffer (6 mM Hepes (pH 7.45), 150 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , and 0.25–1 mCi [ $^{32}\text{P}$ ]phosphate) for 90–120 min at 20–25°C. The incubation buffer was replaced with glucose-free incubation buffer containing the indicated stimuli or inhibitors. At the end of the incubation period the buffer was quickly aspirated and replaced with ice-cold collection buffer (250 mM sucrose, 100 mM NaF, 10 mM EDTA, 2 mM PMSF, 10  $\mu$ g/ml soybean trypsin inhibitor, 10  $\mu$ g/ml leupeptin) to stop cellular metabolism. PMN were scraped from the dishes and total cellular protein was precipitated with 12.5% trichloroacetic acid, extracted with acetone, and dried under nitrogen.

*Polyacrylamide gel electrophoresis and autoradiography.* Both intact PMN and fractions of PMN homogenates were solubilized in sample buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 10% glycerol, 2% SDS, and Bromphenol blue) and

boiled for 3 min. Samples (50–75  $\mu$ g/lane) were subjected to 10% SDS-polyacrylamide electrophoresis [28] using 160  $\times$  200 mm slab gels. Gels were stained with 0.1% Coomassie brilliant blue R-250 and dried, and autoradiography was performed at -70°C for 72 h using Kodak XAR-Omat film. Relative molecular weights were determined with respect to the relative mobility of standard proteins electrophoresed in parallel lanes.

*Quantitation of sn-1,2-diacylglycerols.* Cellular 1,2-diacylglycerols were measured by modifying a previously published method [29]. PMN were treated with inhibitors and stimuli as described above. At the end of the treatment period the tube containing the cells was plunged into an ethanol/solid  $\text{CO}_2$  slush bath and 3 vol. of chloroform/methanol (1:2, v/v) were added to stop the reaction. An additional 1.0 ml of chloroform and 0.5 ml of 1.0 M NaCl were added, the tubes were vortexed, and the mixture was centrifuged at 500  $\times$  g for 5 min to separate the aqueous phase from the lipid-containing organic phase. A 50  $\mu$ l aliquot of the organic phase was dried under nitrogen and resuspended in 20  $\mu$ l of twice-recrystallized octyl  $\beta$ -D-glucoside/cardiolipin solution (7.5%/5 mM in 1.0 DETA?AC). The reaction mixture was completed by adding 50  $\mu$ l of buffer (100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM magnesium, and 1 mM EGTA), 2  $\mu$ l of fresh 100 mM dithiothreitol and 5  $\mu$ l of diglyceride kinase (Lipidex, Middleton, WI). The reaction was initiated by adding 10  $\mu$ l of 10 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP (specific activity: 110000 dpm/nmol) and was allowed to proceed for 30 min at room temperature. The reaction was stopped with 1.0 ml of chloroform/methanol (3:1, v/v) and the phases were partitioned by centrifugation as described. A 500  $\mu$ l aliquot of the organic phase was dried under nitrogen and resuspended in chloroform. A 20  $\mu$ l aliquot of this mixture was spotted onto a Silica Gel 60 chromatography plate (pre-equilibrated with acetone) and was chromatographed using chloroform/methanol/ acetic acid (65:15:5, v/v). The plate was air dried and autoradiography performed for 6 h at -70°C using Kodak XAR-Omat film. Densitometry of autoradiogram was performed using a Bio-Rad or a Helena Quick-Scan R & D densitometer; quantitation of the peak corresponding to 1,2-di-

acylglycerols was accomplished by subtracting background density and nonspecific control density values from test densities. The relevant spots were scraped from wetted silica plates and radioactivity present in the samples was quantitated in 10 ml of Safety Solve (Research Products International, Mount Prospect, IL) by liquid scintillation counting.

## Results

In this study we investigated whether increased protein kinase C activity associated with changes in calcium-induced solubility and increased diacylglycerol levels could be dissociated from increased phosphotransferase activity. In order to measure changes in  $[Ca^{2+}]_i$ , PMN were loaded with fura-2 for 25 min at room temperature, and the change in fluorescence spectra of the probe was measured after the addition of ionomycin, an

ionophore which was used to raise  $[Ca^{2+}]_i$ . The results of these experiments are summarized in Fig. 1. Under our assay conditions the basal  $[Ca^{2+}]_i$  was 65 nM. As little as 50 nM ionomycin caused a significant increase in  $[Ca^{2+}]_i$ , equilibrating at 210 nM after 25 s (Fig. 1, upper tracing). Ionomycin from 50 to 1000 nM caused a dose-dependent increase in  $[Ca^{2+}]_i$ , achieving a maximum of 680 nM at 500 nM ionomycin. Calcium remained elevated within PMN, even after 10 min from the point of addition of ionophore (data not shown). Similar results were obtained when identical measurements were made in loading buffer containing 2 mM EDTA (Fig. 1, lower tracing), but maximal fluorescence changes were about 25% lower at each concentration employed. These results reveal that micromolar concentrations of ionomycin caused a dose-dependent increase in  $[Ca^{2+}]_i$ , which was related to the presence of intracellu-

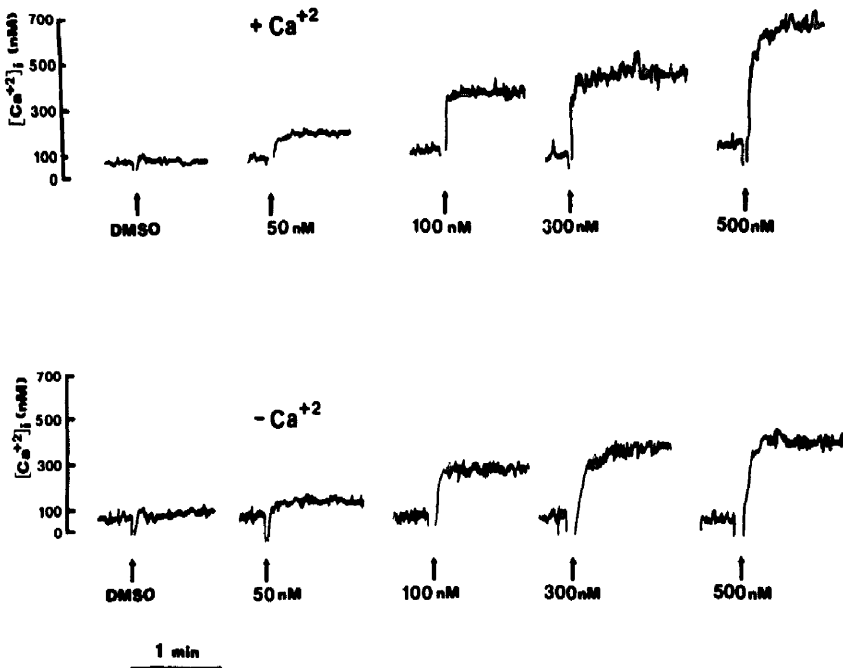


Fig. 1. Changes in intracellular calcium monitored using the fluorescent calcium probe, fura-2. PMN at  $5 \cdot 10^7/\text{ml}$  were loaded with  $2.5 \mu\text{M}$  fura-2/AM at room temperature for 25 min and then washed to remove the unincorporated probe. Fura-2 loaded PMN ( $10^6$ ) in 1 ml loading buffer (upper tracings) or loading buffer without calcium but containing 1 mM EDTA (lower tracings) were mixed with the indicated concentration of ionomycin in a continuously stirring cuvette at  $37^\circ\text{C}$ . Continuous measurements of fura-2 fluorescence changes were made at both 340 and 380 nm. Calcium concentrations indicated on the ordinate axis were derived from direct measurements of the tracings according to the formula described in Materials and Methods.

lar stores, a finding which has been reported [30-32].

Since protein kinase C activity is (by definition)  $\text{Ca}^{2+}$ -dependent, we hypothesized that increased  $[\text{Ca}^{2+}]_i$  could stimulate protein kinase C activity. Heretofore, protein kinase C 'translocation' has been correlated with its activation; translocation has been defined as a relative increase in protein kinase C activity measured in the insoluble fraction of homogenized cells, resulting in that fraction containing most of the cellular protein kinase C activity. This definition is somewhat in error because, as yet, the actual physical mobility of the enzyme from the cytoplasm to the plasma membrane has not been demonstrated (a phenomenon, therefore, which is inconsistent with the term). In fact, the precise subcellular distribution of the enzyme, as opposed to the distribution of enzymatic activity which is measured by the assay, is not known. Therefore, the term 'translocation' will be avoided in the present report in lieu of more specific descriptions.

PMN were incubated with various concentrations of ionomycin for 5 min at  $37^\circ\text{C}$  in order to increase  $[\text{Ca}^{2+}]_i$ . Soluble and insoluble fractions from treated PMN were assayed for protein kinase C activity. Less than 100 nM ionomycin had no significant effect on protein kinase C distribution (Table I), but soluble protein kinase C activity doubled over the short concentration range. At concentrations of 100 nM or greater both increased insoluble protein kinase C activity and also a net loss of soluble activity was observed. Total cellular protein kinase C activity was defined as the sum of the protein kinase C activities recovered from soluble and insoluble fractions from a single ionomycin concentration. Concentrations of ionomycin up to 100 nM stimulated a small increase in total cellular protein kinase C activity (58%). Ionomycin at 300-5000 nM induced a statistically significant ( $P < 0.05$ ) increase in total cellular activity, achieving a doubling at 300 nM. This unusual finding suggests that increased insoluble protein kinase C activity can be induced at concentrations of calcium lower than those which may stimulate activation of the enzyme.

Since increased  $[\text{Ca}^{2+}]_i$  induced changes in the solubility of and quantitative changes in protein

TABLE I

PROTEIN KINASE C ACTIVITY IN SOLUBLE AND INSOLUBLE FRACTIONS OF IONOMYCIN-TREATED HUMAN NEUTROPHILS

PMN were treated with the indicated concentration of ionomycin or vehicle (0.1% DMSO) for 5 min at  $37^\circ\text{C}$ . Treated PMN were homogenized by sonication, and soluble and insoluble fractions were prepared by centrifugation, as described in Materials and Methods. Fractions were subsequently assayed for protein kinase C activity using an in vitro assay and type IHS histone as a substrate. Results are expressed as the means of at least four experiments. S.E. were less than 16% of each given value. In parentheses are the percentages of the total cellular protein kinase C activity (soluble+insoluble activities from the same concentration) represented by that fraction.

[Ionomycin] (nM)	Protein kinase C activity (pmol/min per $10^7$ cells)		
	soluble	insoluble	total
0	227 (69%)	100	327
1	391 (82%)	87	478
10	437 (79%)	111	548
100	91 (17%)	443	534
300	88 (15%)	516	604
500	86 (12%)	619	705
1000	100 (15%)	580	680
5000	90 (13%)	599	689

kinase C activity, we anticipated that increased calcium also induced changes in endogenous cellular phosphoproteins. PMN were loaded with [ $^{32}\text{P}$ ]phosphate, challenged with ionomycin, and subjected to electrophoresis and autoradiography. Phosphate-loaded resting PMN (Fig. 2, lane A) had only about five phosphoproteins visible on autoradiograms. The low level of labeling in resting cells (compared to that observed in previous studies; Refs. 23 and 24) was due primarily to technical changes in the method used to load [ $^{32}\text{P}$ ]phosphate into PMN. Specifically, PMN were loaded in suspension as opposed to being loaded after they had adhered to plastic dishes, because adherence activates them [27]. In addition, PMN were loaded with lower concentrations of radioisotope, for longer incubation times and at lower temperatures than commonly employed using adherence PMN [23,24,27]. These changes resulted in lower background interference on autoradiograms and lower phosphoprotein labeling in resting PMN, but produced more pronounced changes

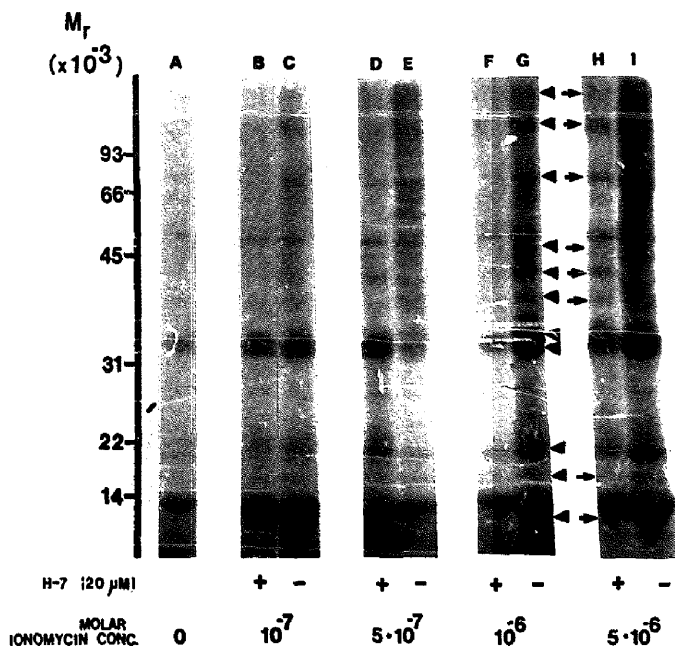


Fig. 2. Phosphoprotein patterns observed on autoradiograms of [ $^{32}\text{P}$ ]phosphate loaded human neutrophils (PMN) treated with ionomycin and H-7. PMN were loaded with [ $^{32}\text{P}$ ]phosphate and treated with 20  $\mu\text{M}$  H-7 (left lanes, +) or buffer (right lanes, -) for 5 min. PMN were subsequently treated with the indicated concentration of ionomycin for 5 min. Total cellular protein was precipitated with trichloroacetic acid, extracted with acetone, and subjected to 10% SDS-PAGE (75  $\mu\text{g}$  protein/lane). Gels were dried and autoradiographed for 72 h at  $-70^\circ\text{C}$ . Data from a single representative experiment, of which three were performed, are shown. The numbers at the right represent the mobility of molecular weight standard proteins. Symbols designating phosphoproteins are explained in the text.

in phosphoproteins on autoradiograms of activated cells.

PMN treated with 100 nM ionomycin for 5 min showed no appreciable changes in phosphoproteins (Fig. 2), but 500 nM or greater concentrations of ionomycin induced increased density of phosphoprotein bands having relative mobilities near 200, 97, 80, 47, 39, 36, 34, 32, 20, 18 and 13 kD (Fig. 2G; arrowheads). Higher concentrations of ionomycin induced denser phosphorylation of existing proteins, and the appearance of several phosphoproteins which were previously unobserved but appeared to be relatively minor constituents. These results indicate that moderate increases in  $[\text{Ca}^{2+}]_i$  induce changes in the solubility of protein kinase C activity, and that higher  $[\text{Ca}^{2+}]_i$  cause changes in cellular phos-

phoproteins which may be a prerequisite for functional activation of PMN.

Since increased calcium concentrations probably activate several calcium-dependent phosphotransferases, we were interested in characterizing which phosphoproteins, if any, were specifically generated by protein kinase C. [ $^{32}\text{P}$ ]Phosphate-loaded PMN were treated with 20  $\mu\text{M}$  H-7 for 5 min, and then incubated with ionomycin. PMN pretreated with H-7 (Fig. 2, left panels) had fewer phosphoproteins than PMN treated without the inhibitor (Fig. 2, right panels); phosphoproteins which were observed at 200, 97, 80, 47, 39, 36, 18 and 13 kD were apparent in H-7 treated cells only at the highest concentration of ionomycin employed (5 · 10<sup>-6</sup> M; Fig. 2H, arrows). H-7 reportedly inhibits protein kinase C in ad-

dition to other enzymes. Therefore, these phosphoprotein changes suggest potential protein kinase C substrates, but do not offer proof because of the lack of specificity of the inhibitor [33]. These results also suggest that changes in protein kinase C activity do not always indicate physiological activation of the enzyme, as defined by the phosphorylation of endogenous substrates.

The activation of protein kinase C *in vivo* requires both calcium and diacylglycerol [7,8]. The results presented above suggest that an identifiable threshold level of intracellular calcium may be required to activate protein kinase C. It has been reported that certain non-steroidal anti-inflammatory drugs inhibit the production of diacylglycerols in monocytes [34], by inhibiting phospholipase C. We hypothesized that if protein kinase C activation required both increased  $[Ca^{2+}]_i$  and increased cellular diacylglycerols, treatment of PMN with anti-inflammatory agents should inhibit protein kinase C activation, but not necessarily the shift in its calcium-induced solubility. PMN were treated with increasing concentrations of either sodium salicylate or acetylsalicylic acid for 5 min. Soluble and insoluble fractions were subsequently assayed for protein kinase C activity. The results of these experiments are summarized in Fig. 3. Acetylsalicylic acid and sodium salicylate inhibited soluble protein kinase C activity in a dose-dependent manner. Insoluble protein kinase C activity from drug-treated PMN was not significant above background activity at any concentration tested. In a parallel study, acetaminophen, ibuprofen, and indomethacin used at similar concentrations had no significant effect on protein kinase C activity (data not shown).

PMN were pre-treated with  $10 \mu M$  acetylsalicylic acid or sodium salicylate for 5 min and then incubated with various concentrations of ionomycin to increase  $[Ca^{2+}]_i$ . Protein kinase C activity in both the soluble and insoluble fractions of these PMN was largely inhibited (Fig. 4), being significant above control levels ( $P < 0.05$ ) only at concentrations greater than  $1 \mu M$  (which also caused a significant amount of lactate dehydrogenase release from the cytoplasm, indicating cell lysis; data not shown). These results suggest that the ionomycin-induced increase in  $[Ca^{2+}]_i$  cannot be solely responsible for increased protein kinase C

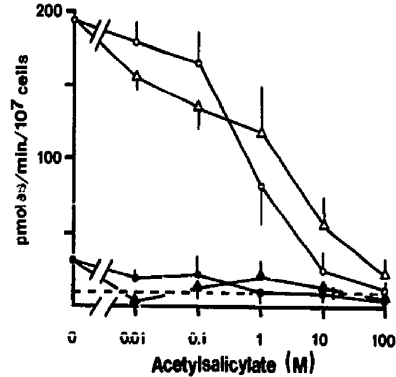


Fig. 3. The effect of anti-inflammatory agents on human neutrophil (PMN) protein kinase C activity. PMN ( $5 \cdot 10^6/ml$ ) were treated with the indicated concentration of acetylsalicylic acid (circles) or sodium salicylate (triangles) for 5 min at  $25^\circ C$ . PMN were disrupted by sonication and soluble (open symbols) and insoluble (closed symbols) fractions were prepared as described in Materials and Methods. Fractions were subsequently assayed for protein kinase C activity as described. Each point represents the mean value from at least three separate experiments. Results are reported as pmol [ $^{32}P$ ]phosphate incorporated into histone per min for  $10^7$  cells,  $\pm S.E.$  The dashed line indicates the level of background phosphotransferase activity in the assay.

activity in human PMN. It is possible that protein kinase C solubility changed but was not measured by our protein kinase C activity assay system. To circumvent this problem, we measured PDB receptors in soluble and insoluble fractions using a PDB-binding assay (Table II). Resting PMN had 92% of their total phorbol receptors associated with the soluble fraction. PMN treated with less than 300 nM ionomycin displayed a similar distribution, but cells treated with 300 nM or greater concentrations of ionomycin had a dose-dependent increase in the relative amount of PDB receptors associated with the insoluble fraction. A maximum of 89% of the total cellular PDB receptors were found in the insoluble fraction using 1000 nM ionomycin. PMN treated with  $1 \mu M$  acetylsalicylic acid and then 500 nM had a distribution of PDB receptors similar to that of cells treated with ionomycin but without acetylsalicylate. These data support the hypothesis that selective non-steroidal anti-inflammatory agents block protein kinase C activation but not changes in calcium-induced solubility. To strengthen our con-



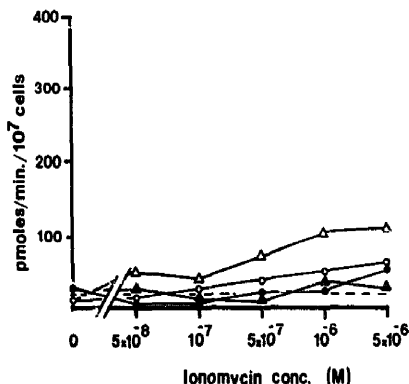


Fig. 4. Effect of ionomycin on aspirin-treated human neutrophils (PMN). PMN ( $5 \cdot 10^6$ /ml) were treated with  $10 \mu\text{M}$  acetylsalicylic acid (circles) or sodium salicylate (triangles) for 5 min at room temperature and then incubated with the indicated concentration of ionomycin for an additional 5 min. Soluble (open circles) and insoluble (closed circles) PMN fractions were prepared and assayed for protein kinase C activity as described in Materials and Methods. Each data point represents the mean of three separate experiments. The results are reported as pmol of [ $^{32}\text{P}$ ]phosphate incorporated into histone protein per min for  $10^7$  cells. S.E. were consistently less than 12% of the reported value. The dashed line indicates the level of background phosphotransferase activity in the assay.

clusion, we measured cellular levels of *sn*-1,2-diacylglycerols after PMN were similarly treated.  $1 \mu\text{M}$  acetylsalicylate depressed the level of 1,2-diacylglycerols in resting cells ( $7 \pm 35\%$  of control, Fig. 5). Ionomycin at  $500 \text{ nM}$  ionomycin stimulated a 3.5-fold increase (compared to vehicle-treated control PMN;  $n = 4$ ) in cellular 1,2-diacylglycerols. PMN treated with  $1 \mu\text{M}$  acetylsalicylate and then ionomycin had levels of 1,2-diacylglycerols comparable to control cells. As expected, cells treated with acetylsalicylate followed by ionomycin and  $1 \mu\text{g/ml}$  OAG had significantly higher levels of 1,2-diacylglycerols ( $220 \pm 29\%$  of control). The results suggest that acetylsalicylate inhibits the ionomycin-induced increase in cellular 1,2-diacylglycerol.

The following experiment was performed to test whether anti-inflammatory agents block protein kinase C activation, measured by endogenous phosphoprotein formation, through an inhibition of 1,2-diacylglycerol production. PMN were loaded with [ $^{32}\text{P}$ ]phosphate and treated with acetylsalicylic acid and ionomycin as described above. PMN

TABLE II

#### DISTRIBUTION OF PHORBOL 12,13-DIBUTYRATE RECEPTORS IN FRACTIONS OF HUMAN NEUTROPHILS

PMN were treated with the indicated concentration of ionomycin or vehicle alone (0.1% DMSO) for 5 min. Some PMN were pre-treated with  $1 \mu\text{M}$  acetylsalicylic acid for 5 min before they were incubated with ionomycin. Soluble and insoluble fractions were prepared and assayed for phorbol ester receptors using a [ $^3\text{H}$ ]phorbol 12,13-dibutyrate-binding assay, as described in Materials and Methods. The data represented the percentage of the total phorbol bound ( $\pm$  S.E.) from at least three separate experiments.

Treatment	% of total [ $^3\text{H}$ ]PDB bound	
	soluble	insoluble
None	$92 \pm 6$	$8 \pm 4$
100 nM ionomycin	$90 \pm 7$	$10 \pm 3$
300 nM ionomycin	$12 \pm 8$	$88 \pm 12$
500 nM ionomycin	$22 \pm 3$	$78 \pm 2$
1000 nM ionomycin	$11 \pm 4$	$89 \pm 9$
$1.0 \mu\text{M}$ acetylsalicylic acid	$86 \pm 9$	$14 \pm 8$
$1.0 \mu\text{M}$ acetylsalicylic acid + 100 nM ionomycin	$88 \pm 6$	$12 \pm 4$
$1.0 \mu\text{M}$ acetylsalicylic acid + 300 nM ionomycin	$9 \pm 2$	$91 \pm 7$
$1.0 \mu\text{M}$ acetylsalicylic acid + 500 nM ionomycin	$17 \pm 8$	$83 \pm 10$

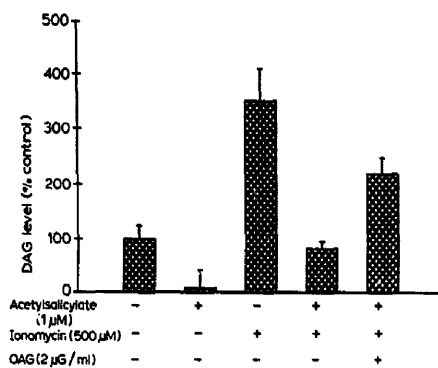


Fig. 5. 1,2-Diacylglycerol (DAG) levels in human neutrophils. Neutrophils were treated for five minutes with  $1 \mu\text{M}$  acetylsalicylate or with buffer. Treated cells were then incubated with either  $500 \mu\text{M}$  ionomycin,  $2 \mu\text{g/ml}$  OAG, or both, for an additional 5 min (cellular treatment is indicated by (+) and buffer treatment in place of an agent is indicated by (-) under each bar). At the end of the incubation period total cellular *sn*-1,2-diacylglycerol levels were quantitated as described in Materials and Methods. Results are expressed as the relative percentage of cellular diacylglycerols with reference to untreated (control) cells and are reported as the mean values ( $\pm 1$  S.D.) from four separate experiments.

were treated with 2  $\mu\text{M}/\text{ml}$  OAG, a synthetic protein kinase C activator [35]. Acetylsalicylate (Fig. 5, lane D) blocked ionomycin-induced phosphorylation (Fig. 5, lane B). In contrast, OAG (Fig. 5, lane E) reversed the acetylsalicylate-dependent inhibition of endogenous phosphoprotein formation. These results suggest that increased  $[\text{Ca}^{2+}]_i$  induces protein kinase C activation and protein phosphorylation by a mechanism which probably requires 1,2-diacylglycerol production.

## Discussion

Activation of protein kinase C in human PMN has been correlated with several specific functional events, including chemotaxis, degranulation,  $\text{O}_2$ -production and protein phosphorylation, all of which are activated during inflammation. Several different classes of agents induce protein kinase C activation, including ligands of surface receptors such as epidermal growth factor [36] and transferrin [11], lipophilic agents such as phorbol esters [4] and 1,2-diacylglycerols [7], and bryostatin 1 [37]. Some of these agents also induce significant changes in cellular phosphoproteins [23,24,27] which may provide a stimulatory signal.

Operationally, protein kinase C 'translocation' has been defined strictly on the basis of its solubility in certain buffers which sometimes contain varying concentrations of detergent and in cells disrupted by at least three different methods, a definition which does not indicate whether protein kinase C actually moves through the cytoplasm to membranes or if its activity is modulated in specific cellular microenvironments. The latter regulatory mechanism may be under the control of protein kinase C-I, the endogenous PMN inhibitor of protein kinase C [24]. Because conflicting reports currently exist, several models have been proposed to explain changes in the solubility of protein kinase C activity. One such model states that stimuli which elevate  $[\text{Ca}^{2+}]_i$  concomitantly increase protein kinase C binding to the lipid components of membranes [38]. Wolf et al. [38] found that the most favorable binding of protein kinase C to membranes occurred at  $[\text{Ca}^{2+}]_i$  approaching 500 nM. Our results agree favorably with those presented in their study indicating that concentrations of ionomycin which increase  $[\text{Ca}^{2+}]_i$  to 500

nM (Fig. 1) also stimulate increased insoluble protein kinase C activity (Table I). Because activating most forms of protein kinase C is dependent upon calcium, we hypothesized that by increasing  $[\text{Ca}^{2+}]_i$  we would increase the enzyme's activity. In our experiments an increase in  $[\text{Ca}^{2+}]_i$  was correlated with higher levels of protein kinase C enzymatic activity. An increase in total activity has been reported [22,39], but this phenomenon is uncharacteristic of protein kinase C activity measurements using subcellular fractions, is highly stimulus-dependent, and most commonly a decrease in activity is observed [4,40]. In support of our data are studies reporting that the calcium-ionophore A23187, although not as specific in its action, stimulated protein kinase C activation in PMN [41] and in monocytes [39]. In their study, Gopalakrishna et al. [42] showed that protein kinase C binding to parietal yolk sac membranes is dependent not only on  $[\text{Ca}^{2+}]_i$  but also upon the stimulus employed. Thus, it appears that increases in  $[\text{Ca}^{2+}]_i$  in some cases may be required to promote protein kinase C binding (or tighter binding) to less soluble cellular structures in order to initiate later functional responses in the PMN which could be activated by protein kinase C-dependent phosphorylation. Our results support that hypothesis because moderate changes in  $[\text{Ca}^{2+}]_i$  increased phorbol-binding in the insoluble fraction but higher concentrations were required to elicit phosphoprotein changes.

Although a calcium/phospholipid-independent form of the protein kinase C has been reported [13,17], we found neither substantial phosphotransferase activity in the absence of calcium, diacylglycerol, and phospholipid, nor increased phorbol-binding in the soluble fraction after PMN treatment with ionomycin or OAG. Therefore, our results do not support the hypothesis that proteolytic cleavage of protein kinase C is required for activation and subsequent phosphoprotein changes.

While the effect of non-steroidal anti-inflammatory drugs on PMN functions such as degranulation and superoxide production are reasonably well documented [43,44], the specific mechanism of action of the drugs has remained elusive. Rationale for the effects of anti-inflammatory drugs in PMN include such explanations as

their being free-radical scavengers [45] and inhibitors of cyclooxygenase activity [46,47]. Bomalski and co-workers [34] have reported that acetylsalicylate inhibited diacylglycerol production through phospholipase C. In our experiments, treatment of PMN with acetylsalicylic acid and sodium salicylate inhibited protein kinase C activity in the insoluble fraction at all concentrations tested, but they inhibited soluble protein kinase C activity in a dose-dependent manner (Fig. 3); this may be explained by the disproportionate amounts of the enzyme in the two fractions. Similarly, these agents inhibited ionomycin-induced protein kinase C activity in a dose-dependent fashion (Fig. 4), suggesting that the inhibition occurs at a point in the pathway which is distal to increased  $[Ca^{2+}]_i$ . Since phospholipase C activity has been reported to reside in the plasma membrane (which is present in our insoluble fraction), it is possible that acetylsalicylic acid and sodium salicylate may inhibit phospholipase C activity and therefore diminish diacylglycerol production. Our data support this hypothesis because ionomycin alone increased 1,2-diacylglycerols 3.5-fold over those in resting PMN, and treatment with acetylsalicylate plus ionomycin resulted in control levels of diacylglycerols (Fig. 5). The implications of these interesting preliminary results are currently under investigation in a more complete fashion, but the results indicate that acetylsalicylate inhibits the mass production of 1,2-diacylglycerol. More importantly, the addition of OAG reversed the effect of acetylsalicylic acid on ionomycin-induced protein kinase C activity and protein phosphorylation (Fig. 6). Based on these results, it is likely that one of the inhibitory effects of acetylsalicylic acid may be through altering diacylglycerol production.

In conclusion, our data indicate that increases in  $[Ca^{2+}]_i$  stimulate changes in the solubility of protein kinase C activity followed by its activation, the latter event being initiated by diacylglycerol and leading to the phosphorylation of endogenous cellular proteins. In support of our observation, Lapetina et al. [48] found that the calcium-ionophore A23187 stimulated the phosphorylation of a 40 kDa platelet substrate of protein kinase C both in the presence and absence of acetylsalicylic acid; whereas ionomycin stimulated neither phospholipase C nor phosphoryla-

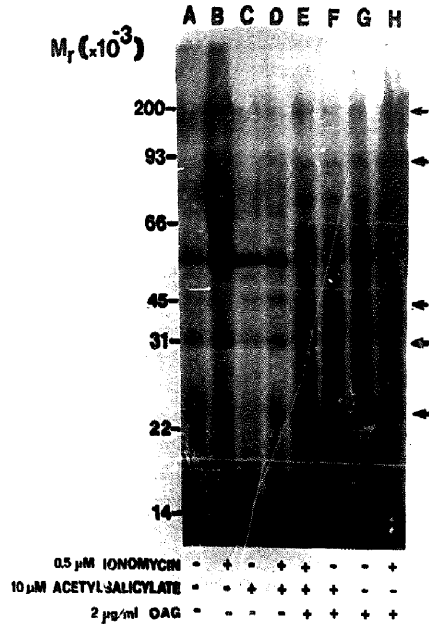


Fig. 6. Phosphoprotein patterns observed on autoradiograms of PMN treated with ionomycin, anti-inflammatory agents, and 1-oleoyl-2-acetylgllycerol (OAG). PMN were loaded with  $[^{32}P]$ phosphate and either pre-treated with 1  $\mu$ M acetylsalicylic acid or vehicle alone (0.1% DMSO) for 5 min (lanes C-F), and subsequently treated with either ionomycin or OAG, or both, for an additional 5 min. Total cellular protein from treated PMN was precipitated with trichloroacetic acid, extracted with acetone, and subjected to 10% SDS-PAGE (160 $\times$ 200 mm gel). Numbers at the left represent the relative mobility of standard proteins electrophoresed in a parallel lane. (A) 0.1% DMSO (control); (B) 0.5  $\mu$ M ionomycin; (C) 1  $\mu$ M acetylsalicylic acid; (D) 1  $\mu$ M acetylsalicylic acid followed by 0.5  $\mu$ M ionomycin; (E) 1  $\mu$ M acetylsalicylic acid followed by 2  $\mu$ g/ml OAG; (G) 2  $\mu$ g/ml OAG; (H) 0.5  $\mu$ M ionomycin followed by 2  $\mu$ g/ml OAG. Arrowheads indicate decreased phosphorylation in proteins from PMN treated with acetylsalicylate before incubation with ionomycin.

tion of the 40 kDa protein in the presence of aspirin in platelets [49]. We also found that ionomycin treatment alone stimulated increased phosphorylation of at least 11 different proteins, of which five were blocked by pretreating PMN with the protein kinase C-inhibitor, H-7 (Fig. 2). Similarly, acetylsalicylic acid inhibited the calcium-dependent phosphorylation of at least five identifiable proteins including those at 200, 92, 45, 31 and 24 kDa which may be protein kinase C

substrates. In support of the notion that protein kinase C activation results in the phosphorylation of unique substrates, we observe that ionomycin and OAG stimulated the phosphorylation of several proteins having molecular masses similar to those inhibited by H-7 and acetylsalicylic acid, including those at 200, 50, 45, 31 and 17 kDa (Figs. 2 and 5) in acetylsalicylic acid-treated PMN. Because OAG stimulates protein kinase C, these results suggest that at least four protein kinase C substrate proteins may be phosphorylated during PMN activation in the absence of a surface ligand. Future work in this area will be required, with special emphasis on more specific inhibitors of protein kinase C when they become available, to detail the steps in cellular functional responses following protein kinase C activation.

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