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# INHIBITION OF NEUTROPHIL SUPEROXIDE FORMATION BY 1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE (H-7), AN INHIBITOR OF PROTEIN KINASE-C

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**Abstract**—Superoxide formation of human neutrophils stimulated by phorbol 12-myristate 13-acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine, or calcium ionophore A23187 was inhibited by pretreatment of the cells with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase-C, but was not inhibited by *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide which has a less inhibitory effect on the protein kinase-C. H-7 also inhibited superoxide formation of PMA-activated cytoplasts, which lack nuclei and granules. The phosphorylation of proteins induced by PMA in the cytoplasts as well as the intact neutrophils was also inhibited by preincubation with H-7. Among several phosphoproteins affected by H-7, one protein with a molecular weight of 19,000 (pI = 4.9) was inhibited markedly. *N*-(2-Guanidinoethyl)-5-isoquinolinesulfonamide did not inhibit the phosphorylation of proteins induced by PMA. These findings support the possibility that the protein kinase-C is involved in the activation process of superoxide formation.

Neutrophils generate superoxide anions ($O_2^-$) during phagocytosis or when stimulated by reagents such as phorbol 12-myristate 13-acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), or calcium ionophore A23187. Superoxide formation is considered to be catalyzed by an NADPH oxidase [1] which is dormant in the resting cells and activated by the stimulants. Intracellular calcium ions [2–5] and the phosphorylation of proteins seem to be involved in the activation [6–10]. The protein kinase-C which is activated by diacylglycerol has been shown to have a crucial role in signal transduction in a variety of cells [11]. We previously suggested the possible involvement of protein kinase-C in the activation process of superoxide formation [12], based on the finding that neutrophils generate superoxide on addition of 1-oleoyl-2-acetyl-glycerol (OAG) which can directly activate the protein kinase-C in intact cells. Synergistic effects between a protein kinase-C activator (OAG or PMA) and a calcium ionophore on superoxide formation have been reported [13, 14]. It was suggested that the activation of the protein kinase-C is sufficient to induce NADPH-oxidase activation because PMA can trigger the NADPH oxidase even when cytosolic free calcium is diminished below the normal resting level [15]. The release of enzymes from neutrophils also seems to be activated by the kinase [16–19].

Several inhibitors known to inhibit the protein kinase-C, such as trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), inhibit superoxide formation of neutrophils, but they are also calmodulin inhibitors. A specific inhibitor of the protein kinase-C is not yet known. Hidaka *et al.* [20] have reported recently that 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of the protein kinase-C. It was shown that H-7 selectively inhibits the phosphorylation mediated by the protein kinase-C in PMA-stimulated platelets [21, 22].

We report in this paper that H-7 inhibits superoxide formation and phosphorylation of proteins in stimulated neutrophils or cytoplasts. These observations are in accord with our previous suggestion that the protein kinase-C is involved in the activation of NADPH oxidase.

## MATERIALS AND METHODS

*Materials.* H-7 and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide were gifts of Prof. Hidaka, Department of Pharmacology, Mie University School of Medicine. PMA, cytochrome *c*, superoxide dismutase, cytochalasin B and sodium dodecyl sulfate (SDS)-molecular weight markers were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). FMLP and Ampholine were from the Peptide Institute (Osaka, Japan) and LKB (Sweden) respectively. $H_3^{32}PO_4$ was obtained from the Nihon Genshiryoku Institute (Tokyo, Japan). Other reagents were of analytical grade.

*Preparation of neutrophils and cytoplasts.* Human neutrophils were prepared from peripheral blood as described previously [5]. The cells were suspended in a buffered saline, pH 7.4, containing 135 mM NaCl, 5 mM KCl, 2 mM glucose and 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid]. Cytoplasts were prepared according to the method of Roos *et al.* [23] as follows. Neutrophils were suspended in a 12.5% (w/v) Ficoll 70 solution con-

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taining cytochalasin B at 5 μg/ml. The suspension was layered on a discontinuous gradient of 16% Ficoll and 25% Ficoll. Cytochalasin B was present throughout the gradient. The gradients were centrifuged for 30 min at 81,000 *g* (middle of the tubes) at 35° in an ultracentrifuge. The band of cytoplasts was harvested from the interface of the 12.5% and 16% Ficoll solutions and resuspended in the buffered saline.

*Assay of superoxide formation.* The superoxide-releasing activity of the cells was measured by the reduction of cytochrome *c*. The assay mixture (1.0 ml) consisted of 75 μM ferricytochrome *c* and $1 \times 10^6$ cells in the buffered saline. After the cells were incubated for 10 min at 37°, the reaction was started by the addition of a stimulant and monitored by a Hitachi dual-wavelength spectrophotometer at 550–540 nm. Superoxide release was calculated from the linear portion of the cytochrome *c* reduction using a molar absorption coefficient of 19,100 $M^{-1}\,cm^{-1}$. NADPH-dependent superoxide formation by the NADPH oxidase was measured at 20° in the assay mixture consisting of 70 μM acetylated cytochrome *c*, 250 μM NADPH and an aliquot of the oxidase (Fraction 2 according to Gabig *et al.* [24]) in buffered saline containing 1 mM $MgCl_2$, pH 7.4.

*Two-dimensional gel-electrophoresis and autoradiography.* Neutrophils or cytoplasts were incubated with about 1 mCi per ml of carrier-free $H_3^{32}PO_4$ for 1 hr at 37° and suspended in the buffered saline. After preincubation, the cells were incubated with a stimulant for 15 min at 37°, and the proteins were extracted by the addition of urea (8.5 M final concentration) and Nonidet-P 40 (2% final concentration). Electrophoresis was performed by the method of O'Farrell with slight modifications [9]. The extracts (400 μg) were subjected to electrophoresis on isoelectric-focusing gels containing 2% Ampholine (pH 3.5–10) for the first dimension and then 13.5% polyacrylamide gels containing SDS for the second dimension. After staining with Coomassie Brilliant Blue, autoradiographs of the gels were obtained at −70° for about a week. The radioactive contents of phosphoproteins were measured by the Joyce Loebl Magiscan-1 image analysis system [25]. The pH gradient formed during electrophoresis in the first dimension was measured according to Cantrell *et al.* [26].

## RESULTS

*Inhibition of superoxide formation by H-7.* On stimulation with PMA (0.1 μg/ml), human neutrophils released superoxide as detected by the reduction of cytochrome *c*. This effect was completely inhibited by superoxide dismutase (SOD, 10 μg/ml) (Fig. 1A, curve a). The maximum activity of superoxide release was 9.7 nmoles/min per $10^6$ cells. Preincubation of cells with 60 μM H-7 led to an inhibition of superoxide formation (Fig. 1A, curve b). The degree of inhibition was essentially equal to that observed when H-7 was added at 3 min after the stimulant (Fig. 1A, curve c). Another derivative of isoquinolinesulfonamide, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (60 μM), which has less inhibitory effect on protein kinase-C did not inhibit superoxide formation (Fig. 1B, curve e). The neutrophils were not damaged by incubation with H-7 as judged by a trypan blue exclusion test and the measurement of extracellular lactate dehydrogenase activity. H-7 did not inhibit the reduction of cytochrome *c* by the xanthine–xanthine oxidase system, indicating that it was not a scavenger of the superoxide anions.

Inhibition of superoxide formation by H-7 ws also observed when the cells were stimulated by FMLP or calcium ionophore A23187, and in the cytoplasts stimulated by PMA. Cytoplasts are a useful tool to study the mechanism of superoxide formation because cytoplasts can produce superoxide but do not contain the exocytic granules, so that we do not

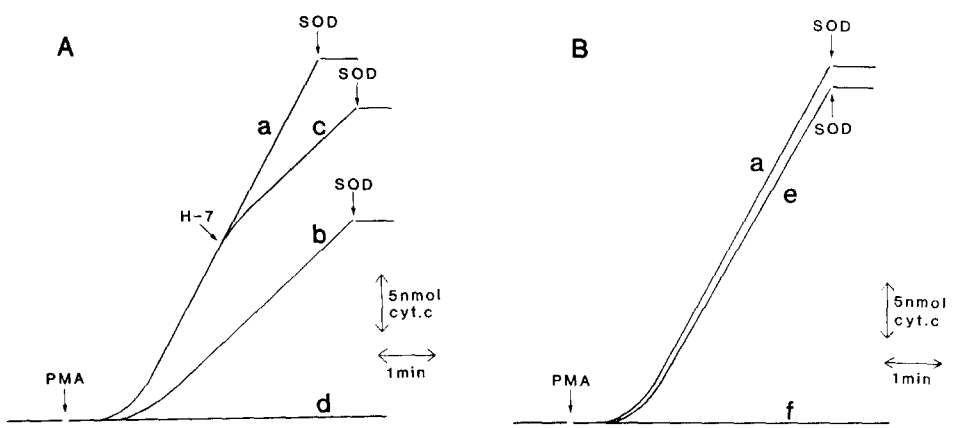


Fig. 1. Time course of superoxide formation in control and H-7 (A) or *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (B) treated neutrophils. Neutrophils were preincubated for 10 min at 37° in a buffered saline and then stimulated by 0.1 μg/ml PMA. Key: (a) control, (b) 60 μM H-7 added 10 min before the stimulation, (c) 60 μM H-7 added at the point indicated by an arrow, (d) 60 μM H-7 added to unstimulated cells, (e) 60 μM *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide added 10 min before the stimulation, and (f) 60 μM *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide added to unstimulated cells.

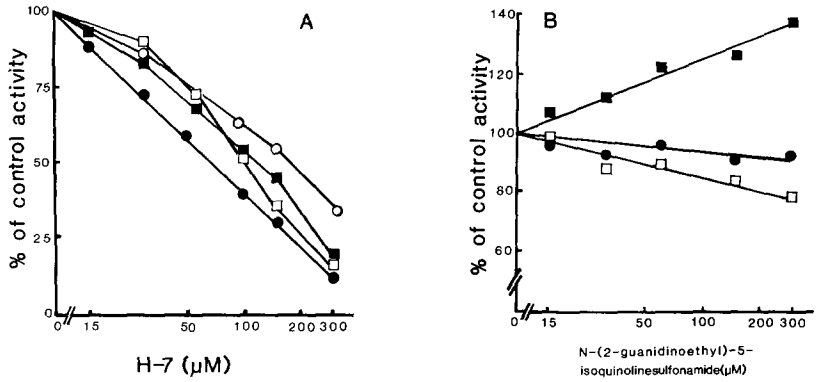


Fig. 2. Effects of H-7 and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide on superoxide formation. Cells were preincubated with an inhibitor at various concentrations for 10 min at 37° in a buffered saline and then stimulated. The reaction mixture contained 1 mM $CaCl_2$ when activated by FMLP or A23187. The results shown were from a single representative of three independent experiments. The activities of superoxide formation are given as the percentage of the control activity. Control activities were as follows: PMA (0.1 μg/ml)-activated neutrophils (●); 9.7 nmoles/min-$10^6$ cells; PMA (0.1 μg/ml)-activated cytoplasts (○): 3.5 nmoles/min/$10^6$ cytoplasts; FMLP (1 μM)-activated neutrophils (■): 5.9 nmoles/min/$10^6$ cells; and A23187 (5 μM)-activated neutrophils (□): 1.5 nmoles/min/$10^6$ cells. (A) H-7; and (B) *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide.

need to consider the effects of enzyme release and the actions of lysosomal proteases. The inhibitory effects of H-7 were dose dependent (Fig. 2A), and the concentrations of H-7 needed for half-maximum inhibition were 68 μM in PMA-activated neutrophils and 180 μM in PMA-activated cytoplasts. In FMLP or A23187-activated neutrophils, they were 120 and 110 μM respectively. *N*-(2-Guanidinoethyl)-5-isoquinolinesulfonamide slightly enhanced superoxide formation induced by FMLP although this compound scarcely affected the formation of superoxide induced by PMA or A23187 (Fig. 2B).

*Inhibition of protein phosphorylation by H-7.* We also studied whether H-7 inhibits the phosphorylation of proteins in neutrophils stimulated by PMA. Cells preloaded with radioactive phosphate were incubated with PMA (1 μg/ml) for 15 min at 37°. The extracts of the cells were subjected to electrophoresis on isoelectric-focusing gels for the first dimension and 13.5% polyacrylamide gels containing sodium dodecyl sulfate for the second dimension, stained with Coomassie Brilliant Blue, and autoradiographed. Figure 3A shows the protein staining pattern of intact neutrophil extracts. These were different from the protein pattern of the cytoplast extracts (Fig. 3B). One difference was the absence of low molecular weight proteins (Fig. 3A, spots a and b) in the cytoplasts as pointed out by Gennaro *et al.* [27]. Another difference was the increased content of several high molecular weight proteins (Fig. 3B, spots c and d) and a protein of $M_r$ = 43,000 (Fig. 3B, spot c) in the cytoplasts. The latter was probably actin judging by the molecular weight and the pI (=5.7). In both neutrophils and cytoplasts, there were no remarkable changes between the stained protein pattern of the resting cells and that of the stimulated ones.

The autoradiographs show that the radioactive contents of several proteins in the stimulated cytoplasts were enhanced compared with those in the resting states (Fig. 4, spots 1–8) and the radioactivity of one spot was decreased (Fig. 4, spot 9). The number of phosphoproteins in the cytoplasts was less than that in the intact neutrophils, but the radioactive contents of the phosphoproteins absent in the cytoplasts did not change in the intact neutrophils on stimulation with PMA. Phosphorylation of a protein [$M_r$ = 19,000, pI = 4.9 (spot 6)] in PMA-stimulated cytoplasts was inhibited markedly by preincubation with 60 μM H-7. The changes in the radioactive contents of several other proteins were also inhibited by preincubation with H-7: they were $M_r$ = 98,000 (spot 1), 49,000 (spot 3), 24,000 (spot 4), 47,000 (spot 7) and 18,500 (spot 9). The radioactive contents of the phosphoproteins measured using Magiscan are summarized in Table 1. These inhibitory effects of H-7 were increased at a higher concentration (300 μM) of H-7 (data not shown). *N*-(2-Guanidinoethyl)-5-isoquinolinesulfonamide (60 μM) did not inhibit the phosphorylation of proteins induced by PMA (data not shown). When the cytosol and the membrane fraction of the sonically disrupted cells were separated by centrifugation at 100,000 *g* for 1 hr, all of the phosphoproteins concerned were found in cytosol and one protein (spot 6) was also found in the membrane fraction (data not shown).

## DISCUSSION

Superoxide formation of PMA-activated neutrophils was inhibited by H-7 but not by *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide. The difference between these two compounds may be due to their abilities to inhibit protein kinase-C because Hidaka *et al.* [20] reported that H-7 is the most potent inhibitor of the kinase ($K_i$ = 6 μM) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide is less effective ($K_i$ = 40 μM), although both compounds

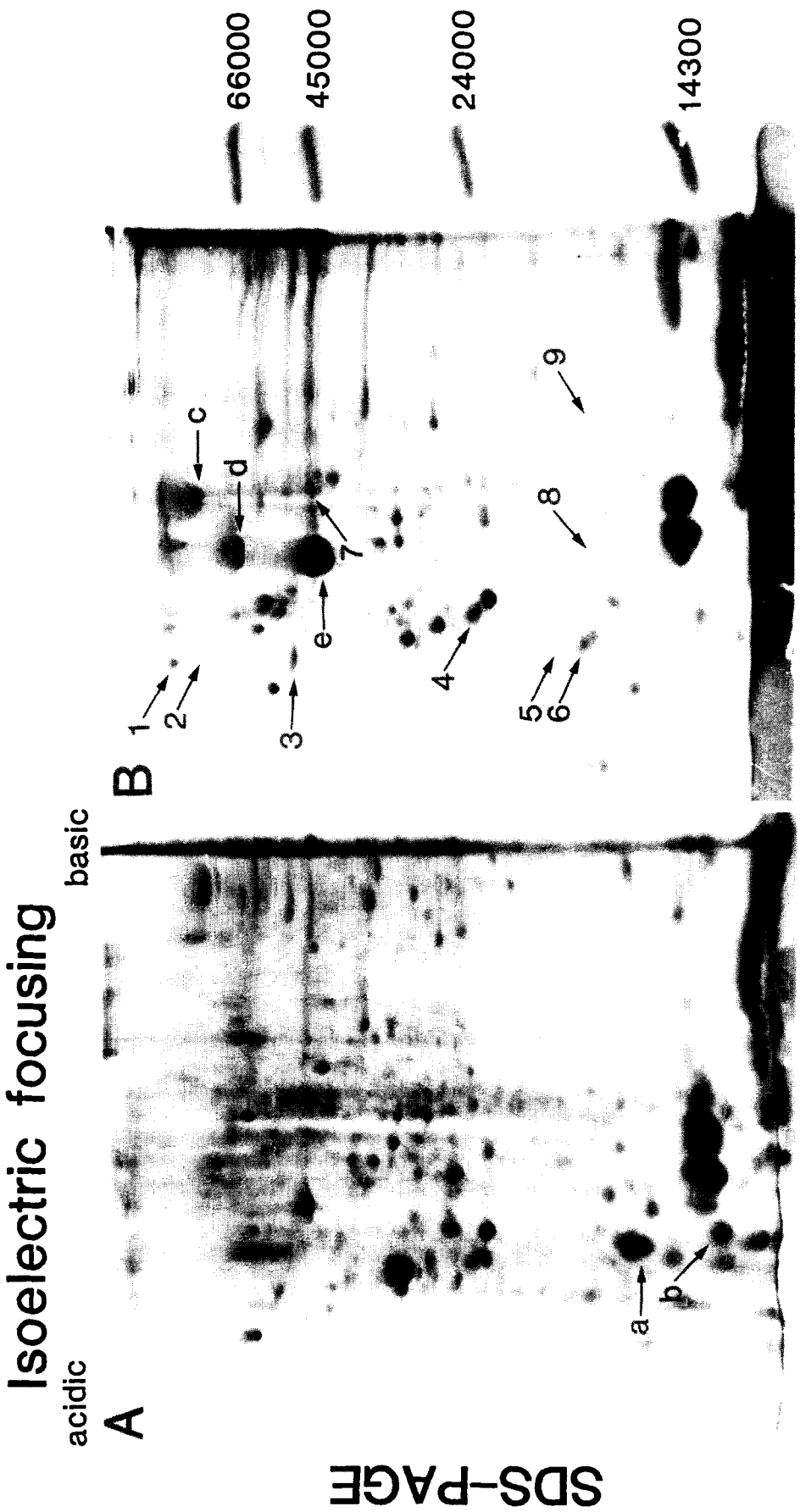


Fig. 3. Electrophoretic profiles of the proteins of intact neutrophils and cytoplasts. Cells were extracted and subjected to electrophoresis on an isoelectric-focusing gel, followed by separation on 13.5% SDS–polyacrylamide gel and staining with Coomassie Brilliant Blue: (A) intact neutrophils, and (B) cytoplasts. Phosphoproteins are indicated by arrows (B, 1–9); the numbers correspond to those of autoradiographs (Fig. 4).

similarly inhibit cAMP- or cGMP-dependent protein kinase and myosin light chain kinase. H-7 inhibits cAMP- or cGMP-dependent protein kinase with a $K_i$ of 3.0 and 5.8 $\mu$M, respectively, but these kinases do not seem to be involved in the process of PMA stimulation because dibutyryl cAMP or cGMP neither induces superoxide formation nor affects the stimulation with PMA [12]. This is further confirmed by the observation that the phosphorylation of proteins in PMA-activated neutrophils is not affected by preincubation with dibutyryl cAMP [10]. Calcium–calmodulin-dependent protein kinase is known to be present in neutrophils [8], but the kinase may not be activated by PMA because PMA does not raise intracellular free calcium at lower concentrations [12]. On stimulation with FMLP or calcium ionophore the kinase may be activated in addition to protein kinase-C, so that the inhibitory effects of H-7 on superoxide formation induced by different stimulants may differ in the concentration of H-7 that produces half-maximal inhibition (Fig. 2A). Superoxide formation enhanced by *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide in FMLP-activated neutrophils (Fig. 2B) may have been due to inhibition of cAMP- or cGMP-dependent protein kinase by this compound, because dibutyryl cAMP or cGMP inhibits the superoxide formation induced by FMLP [12].

The concentration of H-7 necessary for half-maximal inhibition was 68 $\mu$M in the PMA-activated neutrophils. This value is higher than that obtained for the purified protein kinase-C, but it is similar to the concentration (about 50 $\mu$M) for the PMA-induced protein phosphorylation in intact platelets [21]. The concentration of H-7 necessary for cellular inhibition may be higher than the $K_i$ value of H-7 for purified enzyme. This could have been the result of the higher concentration of ATP in neutrophils (7 mM) than in the reaction mixture used for determining the $K_i$ value of H-7 for the protein kinase-C (about 10 $\mu$M) because H-7 is a competitive inhibitor against ATP [20].

Inhibition of the NADPH-oxidase activity by H-7 in Fraction 2 prepared from PMA-activated neutrophils according to the method of Gabig *et al.* [24] was observed, and the $K_i$ value was about 400 $\mu$M (data not shown). Because the $K_i$ value was about six times higher than that of neutrophils (68 $\mu$M), the main site affected by H-7 in intact neutrophils and cytoplasts does not seem to be the NADPH oxidase but one of the activation processes, probably protein kinase-C.

The phosphorylation of proteins in PMA-activated cytoplasts was inhibited by incubation with H-7, and one of the proteins ($M_r$ = 19,000, pI = 4.9) was inhibited markedly by H-7. The proteins ($M_r$ = 49,000, 47,000 and 19,000) we found may correspond with those of the same molecular weight in human neutrophils which Andrews and Babior [28] reported, because the proteins are cytosolic proteins and are phosphorylated by PMA. Gennaro *et al.* [27] reported four proteins in cytoplasts prepared from bovine neutrophils, that are phosphorylated on stimulation with PMA, and they speculated about the involvement of protein kinase-C in the activation process with a protein of $M_r$ = 46,000 as the substrate. Volpi *et al.* [29] also suggested that the same molecular-weight protein in rabbit neutrophils may be the substrate. At present, the substrate of the protein kinase-C in human neutrophils in the activation process of superoxide formation is not known, but six proteins that we found are possible candidates because there is a good correlation between superoxide formation and their phosphorylation in response to PMA and H-7.

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Table 1. Densitometric determination of the radioactive contents of phosphoproteins

| Spot No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| $M_r$ ($\times 10^3$) | 98 | 80 | 49 | 24 | 19.5 | 19 | 47 | 18.5 | 18.5 |
| pI | 4.9 | 4.9 | 4.8 | 5.3 | 4.9 | 4.9 | 7.2 | 5.7 | 6.8 |
| 1. Neutrophils | | | | | | | | | |
| Rest | 7 | 12 | 104 | ND | 72 | 117 | ND | 42 | 174 |
| PMA | 107 | 194 | 235 | ND | 151 | 344 | ND | 89 | 32 |
| PMA + H-7 | 71 | 124 | 134 | ND | 76 | 260 | ND | 92 | 56 |
| 2. Cytoplasts | | | | | | | | | |
| Rest | 254 | 215 | 385 | 3 | 41 | 165 | 8 | 27 | 246 |
| PMA | 380 | 241 | 539 | 41 | 55 | 270 | 79 | 64 | 38 |
| PMA + H-7 | 324 | 281 | 454 | 12 | 41 | 125 | 50 | 65 | 88 |

The number of spots corresponds to that of the spots in Fig. 4. The radioactive contents of phosphoproteins were measured using Magiscan, and values represent means of three independent scannings of the autoradiographs in arbitrary units. ND: not detectable.

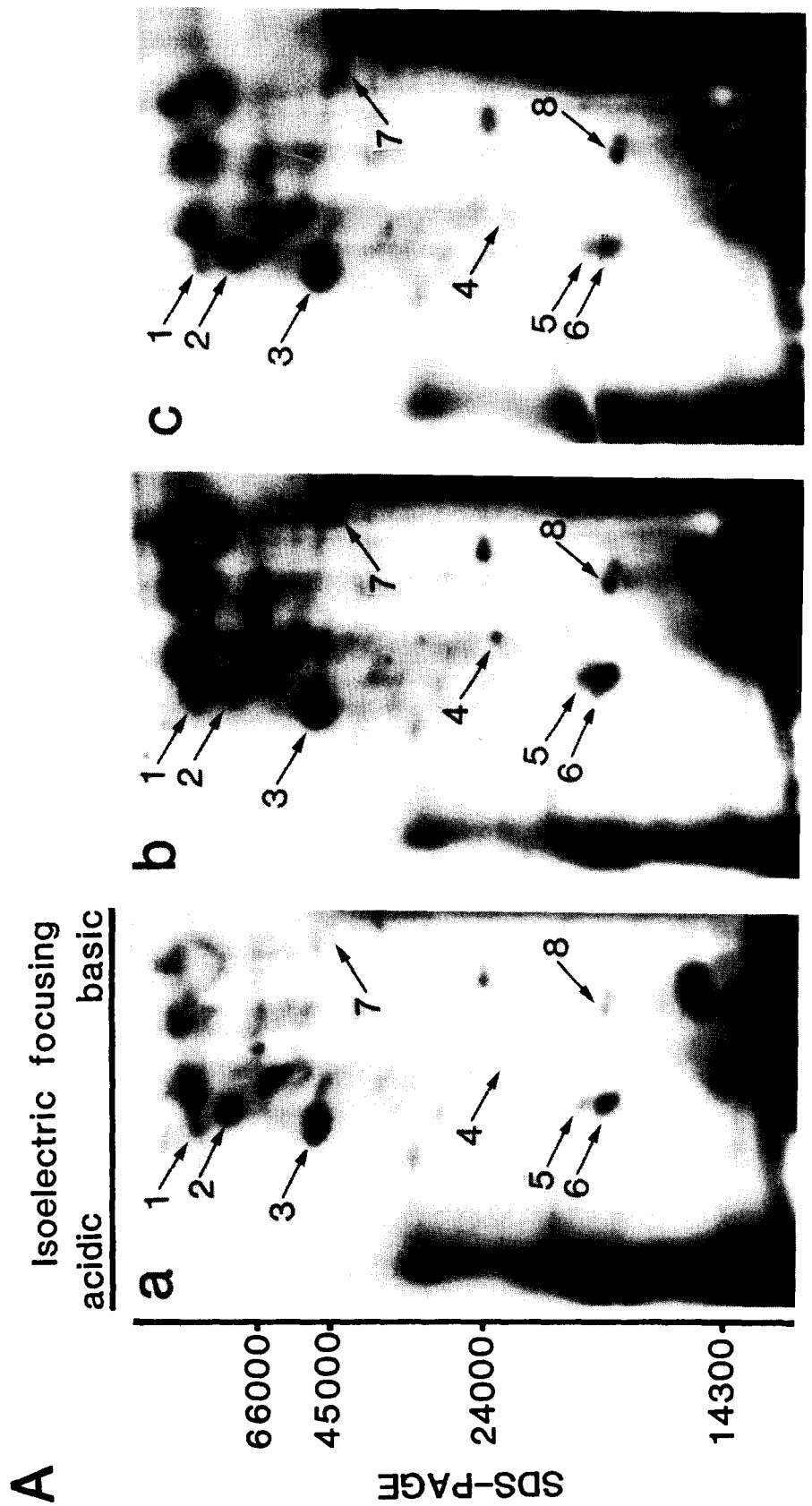
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Fig. 4(a).

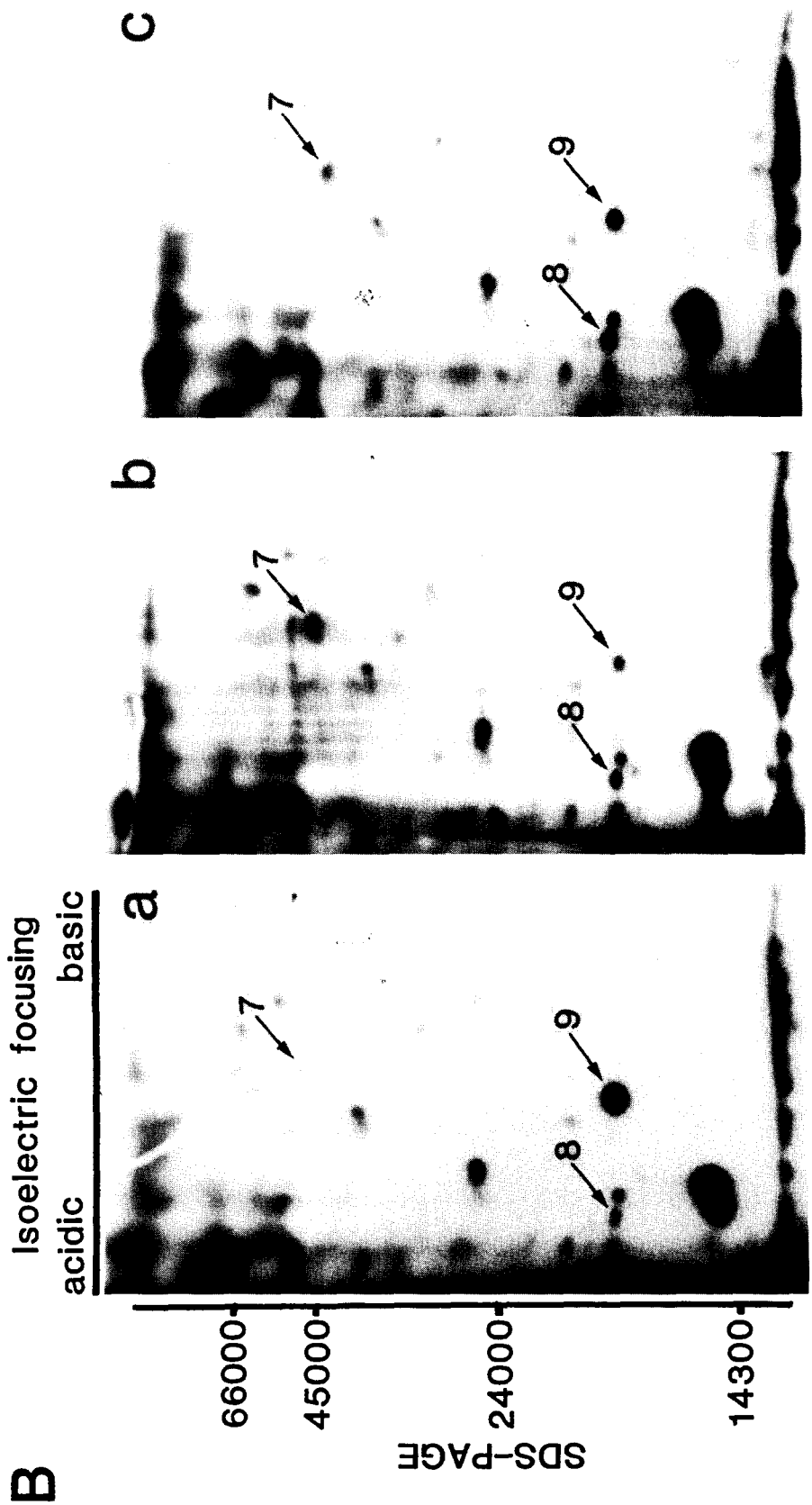


Fig. 4. Effect of H-7 on the phosphorylation pattern of PMA-stimulated cytoplasts. $^{32}$P-loaded cytoplasts were incubated in the absence of (a) and presence of (b) 1 μg/ml PMA or (c) 60 μM H-7 + 1 μg/ml PMA for 15 min at 37°. Two-dimensional gel-electrophoresis and autoradiography were performed as described in Materials and Methods. The same samples of cytoplasts were subjected to electrophoresis on different pH gradients to analyze more acidic proteins (A) and more basic proteins (B).

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