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Negative effect of a protein kinase *C* inhibitor (H-7) on human polymorphonuclear neutrophil locomotion

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SUMMARY

The effects of 1-(5-isoquinoline sulphonyl)-2-methyl piperazine (H-7), a recently described inhibitor of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase *C*), were studied during under-agarose migration of human polymorphonuclear neutrophils (PMN) stimulated by various chemoattractants, in order to determine whether protein kinase *C* is involved in PMN locomotion. The effect of H-7 on the oxidative burst induced by phorbol 12-myristate 13-acetate or *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) was also measured. Pre-incubation of PMN with H-7 concentrations ranging from 50 to 400 μM inhibited: (i) spontaneous PMN migration under agarose; (ii) the directed migration induced by activated serum, leukotriene B4 or FMLP; and (iii) the speed of the migration induced by FMLP. The inhibition by H-7 of FMLP-induced directed migration was less when FMLP was used at high concentrations which, in the absence of H-7, inhibit locomotion. H-7 depressed the oxidative burst induced by phorbol myristate acetate (PMA) but not that induced by FMLP. All the effects of H-7 on the oxidative burst and migration were reversed by washing PMN after H-7 treatment. These findings indicate that protein kinase *C*, inhibitable by H-7, is involved in a mechanism controlling the speed of PMN locomotion.

INTRODUCTION

Chemotactic factors, such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), complement-derived C5a or leukotriene B4 (LTB4), possess specific membrane receptors on human polymorphonuclear neutrophils (PMN). Relatively low concentrations of these factors are required to induce optimal migration of PMN, but higher concentrations are needed to stimulate their optimal respiratory burst or degranulation (Snyderman & Pike, 1984). There is a contrast between the large number of studies devoted to the mechanisms by which chemotactic receptors trigger PMN respiratory burst or degranulation, and the few concerning migration, particularly the involvement of the Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Snyderman, Smith & Verghese, 1986; Rossi, 1986; Tauber, 1987). PKC is a protein-phosphorylating enzyme that is activated by endogenous diacylglycerol (Kaibuchi *et al.*, 1983) or exogenous phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) (Nishizuka, 1984). The latter is a potent stimulant of the oxidative burst but has no chemotactic effect on locomotion. There is considerable evidence that PMA exerts its effect by activating PKC (Tauber, 1987; Berkow, Dodson & Kraft, 1987). This includes the inhibition of PMA-induced oxidative burst by antagonist of PKC. It has also been reported that in PMN, a high concentration of FMLP induces translocation of PKC (Nishihira, McPhail & O'Flaherty, 1986), thus suggesting that it is involved in this type of stimulation. However, the results of pharmacological studies using an antagonist of PKC suggest that it does not control PMN activation by FMLP because respiratory burst induced by the peptide was not altered by the antagonist (Berkow, Dodson & Kraft, 1986). In order to ascertain whether or not protein kinase *C* is involved in spontaneous and/or directed PMN migration, we measured the effect of a recently described inhibitor of PKC, 1-5-isoquinoline sulphonyl)-2-methyl piperazine, H-7, on this migration, which was assayed under agarose. The results indicated that H-7 slows down both spontaneous and stimulated migration.

Abbreviations: C-1, l-(15-isoquinoline sulphonyl)piperazine; DMSO, dimethylsulphoxide; FMLP, formyl-methionyl-leucyl-phenylalanine; H-7, l-(5-isoquinoline sulphonyl)-2-methyl piperazine; KRP, 0·15 M Krebs' ringer phosphate buffer, pH 7·4; LTB4, leukotriene B4; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil.

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MATERIALS AND METHODS

Reagents

H-7 (Calbiochem, Paris) and superoxide dismutase (Sigma Chemical Co., St Louis, MO) were dissolved in saline and stored in aliquots at $-80°$ at the concentrations of 10^{-2}M and 1 mg/ml,

respectively. FMLP, PMA, cytochalasin B (Sigma, St Louis, MO) and LTB4 (a gift from Laboratoire Hoechst, Paris) were dissolved in dimethylsulphoxide (DMSO) at concentrations of 10^{-2}M, 1 mg/ml, 1 mg/ml and 10^{-4}M, respectively, and stored in aliquots at −80°. The final concentration of DMSO in the incubation medium was less than 0·5% and had no effect on PMN functions. Sera from several healthy volunteers were pooled and stored in aliquots at −80°.

Isolation of PMN

Blood was obtained from the peripheral vein of healthy adults, in preservative-free lithium heparin (10 IU/ml of blood). PMN were isolated by one-step centrifugation of the blood on a mixture of Ficoll–Hypaque (Monopoly resolving medium, Flow Laboratories, France), as previously described (Ferrante & Thong, 1980). Contaminating erythrocytes were removed by one hypotonic lysis (30 seconds). The final leucocyte suspension was diluted in 0·15 M Krebs ringer phosphate, pH 7·4 (KRP), and contained about 98% PMN.

Superoxide anion production

Superoxide anion production by PMN stimulated by FMLP or PMA was measured according to Cohen, Chovaniec & Davies (1980) by continuous recording of cytochrome *C* reduction with a Uvikon 810/820 spectrophotometer, as reported previously (Périanin *et al.*, 1987). Briefly, reaction mixtures containing 10^6 PMN in KRP and 80 μM cytochrome *C* were preincubated in plastic microcuvettes for 10 min at 37° in the presence of 100 μM H-7 or in its absence (control). Cytochalasin B (5 μg/ml) was then added to the medium. Five minutes later, PMN stimulation was triggered by adding either 10^{-6}M FMLP or PMA (0·1 μg/ml) to the cuvettes, and cytochrome *C* reduction was recorded for 10 min. Superoxide dismutase (20 μg/ml) was used to assess the reduction of cytochrome *C* independent of O_2. Results are expressed in nmole O_2 per 10^6 PMN per min, and were calculated under initial rate conditions, i.e. from the linear part of the curve.

Migration assays

PMN migration was measured under agarose in two different ways: the first method served to assess directed migration (i.e. both its speed and orientation) in the presence of FMLP, LTB4 or serum, placed in chemoattractant wells, as previously described (Périanin, Labro & Hakim, 1982). The second method was used to measure speed of migration only, either spontaneous, i.e. in the absence of chemokinetic factors, or stimulated in the presence of these factors, which were incorporated into the agarose gel, as previously reported (Périanin *et al.*, 1985) Five microlitres of PMN suspension (i.e. 5×10^5 PMN) were placed in each well and incubated for 15 min at 37° either in the absence of H-7 (control), or in the presence of H-7 concentrations ranging from 25 to 400 μM. Spontaneous and directed PMN migration were evaluated by the front lead of PMN migration (at least 10 PMN), as previously reported (Périanin *et al.*, 1982, 1985).

Statistics

The significance of differences between the results of experiments performed in the presence of H-7 and in its absence (control) was assessed by the paired Student's *t*-test and *P* values below 0·05 were considered significant.

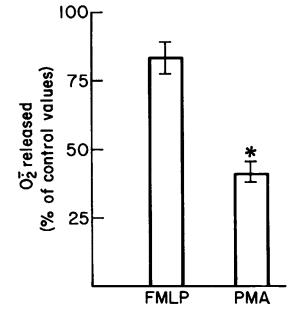

Figure 1. Effect of H-7 on the PMN release of superoxide anion (O_2^-) induced by FMLP an PMA. Results, in the presence of H-7, are expressed as percentage of the O_2^- release by the control, i.e. measured in the absence of H-7. These were 91·3±9 and 41·3±4% for PMN stimulated by FMLP (10^{-6}M) and PMA (100 ng/ml), respectively. Control values were: 9·7±0·8 nmoles $O_2^-/10^6$ PMN/min for FMLP and 6·8±0·45 nmoles $O_2^-/10^6$ PMN/min for PMA. Values are mean±1 SD of three experiments, each conducted in duplicate.

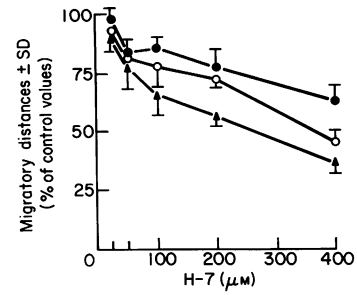

Figure 2. Dose-related effect of H-7 on spontaneous PMN migration and on directed migration induced by serum or LTB4. Results are expressed as percentage of control migratory distances. Values for spontaneous migration (▲) and for migration induced by $2 \cdot 10^{-7}$M LTB4 (●) and by serum (○) after 2 hr incubation were 0·64±0·14 mm, 1·60±0·25 and 1·80±0·16, respectively (mean±1 SD, $n = 5$–7).

RESULTS

Differential effects of H-7 on superoxide anion production by PMN

The results in Fig. 1 confirm that preincubation of PMN with 100 μM H-7 for 15 min at 37° strongly inhibits the rate of superoxide anion production when PMN are stimulated by PMA ($P < 0{\cdot}05$). In contrast, when the chemotactic factor FMLP was used as stimulant, this production was not significantly altered. The inhibition of the burst induced by PMA was completely reversed when PMN were preincubated with H-7 and washed twice. These results indicate that H-7 is not cytotoxic and are consistent with previous data indicating that PKC controls the PMN activation induced by PMA but not that induced by FMLP (Berkow *et al.*, 1986, 1987).

Inhibitory effect of H-7 on spontaneous and directed PMN migration

Pre-incubation of the PMN with various concentrations of H-7 added to the incubation medium inhibited spontaneous PMN

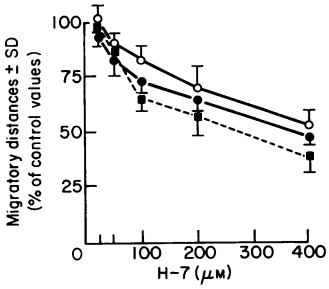

Figure 3. Dose-related effect of H-7 on the directed PMN migration induced by three concentrations of FMLP. Migration distances were assessed after 90 min incubation at 37°. Results are expressed as the percentage of the corresponding control migratory distances which were $0{\cdot}90 \pm 0{\cdot}20$ mm for 10^{-8}M FMLP (■), $1{\cdot}92 \pm 0{\cdot}27$ for 10^{-7}M (●) and $1{\cdot}20 \pm 0{\cdot}16$ for 10^{-6}M (○) (mean ± 1 SD, $n = 5$–7).

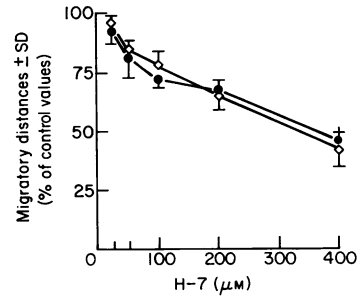

Figure 4. Dose-related effect of H-7 on the speed of migration induced by FMLP. FMLP was incorporated into the agarose gel at the optimal and suboptimal concentrations of 10^{-8}M (●) and 10^{-9}M FMLP (◊), respectively. Control values were $2{\cdot}08 \pm 0{\cdot}08$ mm (10^{-8}M) and $1{\cdot}26 \pm 0{\cdot}24$ (10^{-9}M) for 90 min incubation. Assay values are expressed as percentage of control values.

Table 1. Spontaneous and directed migration of PMN pretreated with H-7 and whether or not further washed

	Migration distances (% of control values)	
Chemoattractant	Unwashed PMN	Washed PMN
KRP	38 ± 2	67 ± 6
10^{-8}M FMLP	36 ± 5	74 ± 7
10^{-7}M FMLP	45 ± 5	80 ± 4
10^{-6}M FMLP	62 ± 7	87 ± 8
Serum	53 ± 7	78 ± 3
$2{\cdot}10^{-7}$M LTB4	47 ± 2	79 ± 14

PMN were pretreated for 15 min at 37° with 400 μM H-7, and 5 μl of PMN suspension were placed in the PMN wells. Aliquots of these pretreated PMN were then washed twice in KRP and PMN migration assayed. Each bar represents the mean ± 1 SD of four to five experiments. Results are expressed as the percentages of the corresponding control values for untreated PMN.

migration in a dose-dependent manner (Fig. 2). For H-7 concentrations of up to 50 μM, the inhibition was statistically significant (i.e. 38 ± 13% of control values, mean ± SD of five experiments, $P < 0{\cdot}05$). The H-7 concentration required for half inhibition of spontaneous migration (IC50) was approximately 300 μM. As expected from this result, H-7 also impaired directed PMN migration: this is illustrated in Fig. 2, which shows that the directed migrations induced respectively by LTB4 and serum were both inhibited in a dose-dependent manner by H-7, with similar IC50 values of about 350 μM. In the presence of FMLP, H-7 concentrations up to 50 μM also inhibited in a dose-dependent manner the directed PMN migrations induced by the suboptimal, optimal and deactivating FMLP concentrations of 10^{-8}, 10^{-7} and 10^{-6}M, respectively (Fig. 3). However, this inhibition was inversely proportional to the FMLP concentrations, since IC50 values were observed for H-7 concentrations of 250, 350 and 400 μM with FMLP concentrations of 10^{-8}, 10^{-7} and 10^{-6}M, respectively. The inhibition by H-7 of both spontaneous and stimulated PMN migration was not due to any incidental cytotoxic properties of H-7, because a similar proportion of PMN (more than 95%) excluded trypan blue, whether they were incubated with high H-7 concentrations of 200 or 400 μM or in the absence of H-7 (controls). The absence of cytotoxic effects after H-7 treatment was also confirmed by the observation that the PMN preincubated with 400 μM H-7 and then washed twice displayed larger spontaneous and directed migration ($P < 0{\cdot}05$) than unwashed cells treated with H-7 (Table 1). This result also suggests that the cell modifications by which H-7 inhibits PMN responses are reversible.

Inhibition by H-7 of the speed of FMLP-induced PMN migration

To investigate the effect of H-7 on the speed of PMN migration, i.e. chemokinesis, we measured its action on the migration induced when a uniform concentration of FMLP was incorporated into the agarose gel, i.e. in the absence of formation of a concentration gradient of FMLP (Périanin *et al.*, 1985). The results in Fig. 4 confirm that H-7 concentrations of up to 50 μM inhibited, in a dose-dependent manner, the stimulating effect of FMLP on the speed of migration. Under optimal PMN stimulation by FMLP, the IC50 of H-7 for speed of migration was about 250 μM.

DISCUSSION

The results reported in this paper show that preincubation of PMN with H-7 inhibits both the spontaneous and directed PMN migration induced by LTB4, activated serum or FMLP. They also indicate that the inhibition of this migration by H-7 is mainly chemokinetic, and can be partially reversed, either by increasing the chemotactic factor concentration or by washing the cells pretreated with H-7. The mechanisms by which H-7 alter PMN functions probably occur though the inhibition of PKC. This hypothesis is supported by direct and indirect evidence. For instance, H-7 has been shown to inhibit directly PKC activity with a relative selectivity since its Ki value for PKC is 6 μM whereas its Ki value for calmodulin-dependent kinase is 97 μM (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984). It was also shown that H-7 inhibited the PMN respiratory burst induced by PMA, a compound which directly binds to PKC

(Castagna *et al.*, 1982) and induces its translocation from the cytosol to the PMN membrane (Wolfson *et al.*, 1985). H-7 also inhibits the phosphorylation in PMN of a 50,000 MW protein associated with kinase *C* activation (Sha'afi *et al.*, 1986). Furthermore, H-7 reverses the inhibition induced by PMA of FMLP-stimulated PMN responses such as respiratory burst, enzyme release or rise in intracellular calcium (Berkow *et al.*, 1987; Sh'afi *et al.*, 1986). These latter data strongly suggest a role of PKC in the regulation rather than in the initiation of PMN functions. Nevertheless, it is still not clear whether PKC activity is involved in the mechanisms activating and/or regulating PMN functions such as locomotion, respiratory burst or the lysomal enzyme release induced by FMLP. Thus, it was recently reported that potent PKC antagonists impaired the PMN respiratory burst induced by PMA but not that induced by FMLP (Berkow *et al.*, 1986, 1987), suggesting that PKC does not control FMLP-induced PMN activation. On the other hand, a recent abstract indicated that C1[1-(5 isoquinoline sulphonyl) piperazine], which inhibits PKC, depressed FMLP-induced migration of PMN, assessed in a Boyden chamber (Harvath *et al.*, 1986). In an attempt to substantiate the latter point, we used a different technique here (under-agarose technique) to analyse the effects of H-7 on this migration. Our results firstly confirm that the pretreatment of PMN with 100 μM H-7 depresses PMA-induced respiratory burst by approximately 50%. The high concentrations of H-7 needed to inhibit PMA-induced oxidative burst may be due, as previously suggested by Berkow *et al.* (1987), to a poor ability of H-7 to enter the PMN. With regard to locomotion, H-7 inhibited spontaneous PMN migration in a dose-dependent manner. However, greater concentrations of H-7 were required to inhibit PMN migration in comparison to respiratory burst. This may be due to the fact that once H-7 was placed into the PMN wells together with PMN, its concentration declined due to the diffusion of the drug under agarose. The impairment of PMN spontaneous migration by H-7 disagrees with the previous finding (Harvath *et al.*, 1986) that spontaneous PMN migration is not altered by the PKC inhibitor C-1. This discrepancy may be due to basic differences between the two migration assay methods. One important difference is that the agarose technique allows PMN to migrate over much longer distances than the Boyden chamber method. Another difference which might account for the discrepant results is that here H-7 was used as PKC inhibitor, whereas C-1 was used in the Boyden chamber method. It is possible that the differences between the effects of H-7 and its demethylated derivative (C-1) are due to differences in the ability of these drugs to enter PMN. If, under our experimental conditions, H-7 really inhibited PKC, as the impairment of the PMN respiratory burst induced by PMA seems to indicate (Fig. 1), this does suggest that PKC inhibitable by H-7 is involved in the mechanisms governing basal PMN migration. This hypothesis seems compatible with the observation that proteins involved in cytoskeletal assembly such as myosin, vinculin and filamin can serve as substrates for PKC (Katoh, Wise & Kuo, 1983; White *et al.*, 1984; Kawamoto & Hidaka, 1984). The impairment of spontaneous PMN migration by H-7 implies that migration stimulated by different chemoattractants is also impaired. This was confirmed here for three important chemoattractants: FMLP (Figs 3 and 4), LTB4 and activated serum (Fig. 2). The observation that the inhibitory effect of H-7 on FMLP-induced PMN migration was partially reversed by increasing the FMLP concentrations suggests that PKC may be involved in PMN stimulation by FMLP.

In conclusion, the protein kinase *C* inhibitor H-7 inhibited both the spontaneous and stimulated PMN migration induced by three chemotactic factors, by slowing down this migration. Thus, PKC (H-7-inhibitable) is probably involved in the regulation of locomotion speed. Further studies are needed to identify the protein(s) whose phosphorylation is (are) involved in migration speed.

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