Rapid Paper**Identification of Distinct Pathways for Superoxide Generation in Mouse Macrophages: An Investigation Using a Chemiluminescence Probe Specific for Superoxide Anion**

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Dependency of superoxide anion (O_2^-) generation on protein kinase C (PKC) or calmodulin (CaM) in macrophages (MPs) was investigated using a O_2^- -specific chemiluminescence probe with the *Cypridina* luciferin analog 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]-pyrazin-3-one (MCLA). In opsonized zymosan (OZ)-stimulated MPs, 40% of the MCLA-dependent chemiluminescence disappeared when the PKC inhibitor 1-(5-isoquinolylsulfonyl)-2-methylpiperazine (H-7) or the CaM antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was added. In MPs stimulated by 12-*o*-tetradecanoylphorbol-13-acetate (TPA), addition of H-7 strikingly lowered MCLA-dependent chemiluminescence, but W-7 had only a little effect. These results indicate that both PKC and CaM are involved in OZ-stimulated O_2^- generation in MPs and that TPA-stimulated O_2^- generation depends not on CaM but on PKC.

Macrophages (MPs), as well as polymorphonuclear neutrophils (PMs), function as effector cells for host defense. During phagocytosis, or upon appropriate stimulation, MPs produce substantial quantities of superoxide anion (O_2^-).^{1,2)} In the generation of O_2^- in MPs and PMNs, protein kinase C (PKC) and/or calmodulin (CaM) have been reported to be

involved,^{3,4)} and it has been thought that different types of stimuli take their own multi-step pathways at first but that finally share a common step to activate the O_2^- -generating enzyme system NADPH oxidase.^{5,6)}

O_2^- is usually monitored by the reduction of ferricytochrome *c*.⁷⁾ However, although this method is highly sensitive for the detection of O_2^- , this method is complicated and requires large amounts of samples,⁸⁾ and strictly, is not specific to O_2^- . O_2^- from phagocytic cells cannot be detected by luminol-dependent chemiluminescence, a widely used method for the detection of reactive oxygen species.^{9,10)} Recently, Nakano *et al.*¹¹⁾ reported that a chemiluminescent probe with the *Cypridina* luciferin analog, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA), respond specifically to O_2^- and is very useful for investigating the ability of O_2^- generation by MPs.

In this paper, we investigated the real dependency of O_2^- generation in mouse MPs on PKC and CaM using a simple and O_2^- -specific MCLA-dependent chemiluminescence method.

Materials and Methods

Proteose peptone-elicited peritoneal MPs were harvested from 6-week-old male C3H/He mice 4 days after intraperitoneal injection of 1.0 ml of 10% proteose peptone (Difco). Cells from 6 to 10 mice were pooled, washed, and counted on a hemocytometer. These preparations contained >80% MPs as measured by differential cell counts. The cell suspensions containing 5×10^5 MPs were plated on a plastic dish (Falcon 1008) and incubated for 60 min at 37°C in 5% CO₂, then washed to remove non-adherent cells. MPs were washed with phenol-red-free Hanks' balanced salt solution, pH 7.40, just before the chemiluminescence measurement.

Chemiluminescence was measured at 37°C using a single photoelectron counting system originally constructed by our project using a photomultiplier tube (Hamamatsu R1333) and a photon counter (Stanford SR-400). In all experiments, 5×10^5 MPs were used in a total volume of 2.0 ml in the presence of either MCLA (500 nM, a generous gift from Dr. M. Nakano, College of Medical Care and Technology, Gunma University, Gunma, Japan) or luminol (30 μ M, Wako). To stimulate respiratory bursts of MPs, both the particulate stimulus opsonized zymosan

(OZ, 0.5 mg/ml) and soluble stimulus 12-*o*-tetradecanoylphorbol-13-acetate (TPA, 100 ng/ml) were used. OZ was prepared as described by Johnston *et al.*¹²⁾ To investigate the dependency of MPs on PKC or CaM, the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7, 50 μ M) and the CaM antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, 50 μ M) were used. *N*-(2-Guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), the control compounds for H-7 and W-7, respectively, were also checked at the same concentration. H-7, W-7, HA-1004, and W-5 were obtained from Seikagaku Kogyo. As MCLA responds not only to O_2^- but also to singlet oxygen (1O_2),¹¹⁾ the influence of a scavenger of 1O_2 , NaN_3 ¹³⁾ (0.5 mM) was also checked. The results were compared by Student's *t*-test.

Results and Discussion

We investigated the dependency on the PKC and CaM for the generation of O_2^- in MPs. Typical patterns of MCLA- and luminol-dependent chemiluminescence are shown in Fig. 1, and the peak chemiluminescence intensities are summarized in Table I. The addition of NaN_3 had no influence on the luminescence response, indicating that the involvement of 1O_2 could be neglected. No change in the chemiluminescence intensity was observed

when HA-1004 or W-5 was added, showing that H-7 or W-7 specifically inhibits PKC activity or CaM function, respectively.^{14,15)} Our preliminary experiments showed that H-7 and W-7, together with HA-1004 and W-5, had no quenching effect on O_2^- generated by the hypoxanthine-xanthine oxidase reaction.¹⁶⁾ Chemiluminescence intensities of OZ-stimulated MPs decreased to some extent in the presence of H-7 or W-7 in both MCLA- and luminol-dependent chemiluminescence (Fig. 1b and d). These results indicate that both PKC and CaM are involved in enhancing the O_2^- generation in MPs, but that the O_2^- generation in MPs does not completely depend on PKC nor CaM when the cells are stimulated by OZ. On the contrary, in TPA-stimulated MPs (Fig. 1a and c), O_2^- -specific MCLA-dependent chemiluminescence intensities strikingly decreased when H-7 was added, but the addition of W-7 had only a little effect on the chemiluminescence intensity. On the contrary, O_2^- -unspecific luminol-dependent chemiluminescence decreased to some extent not only when H-7 was added but also when W-7 was added. The results shown in Fig. 1a clearly demonstrate that the gener-

Fig. 1. Typical Patterns of (a, b) MCLA- and (c, d) Luminol-dependent Chemiluminescence of Macrophages Stimulated by (a, c) TPA and (b, d) OZ.

Experimental conditions are shown in **Materials and Methods**. Results are expressed as the single measurement of luminescence in one of the three representative experiments. 1, unstimulated; 2, stimulated; 3, stimulated + NaN_3 ; 4, stimulated + H-7; 5, stimulated + W-7; 6, stimulated + HA-1004; 7, stimulated + W-5.

Table I. CHEMILUMINESCENCE OF TPA- OR OZ-STIMULATED MACROPHAGES

Treatment	Peak chemiluminescence intensities (counts/sec)	
	MCLA-dependent	Luminol-dependent
(TPA-stimulated macrophages)		
Unstimulated	25,325 ± 402*	219 ± 2*
Stimulated	218,107 ± 4,166	1,367 ± 10
+ H-7	96,189 ± 1,016*	890 ± 9*
+ W-7	190,774 ± 759**	798 ± 27*
+ HA-1004	224,172 ± 2,498	1,264 ± 46
+ W-5	208,099 ± 12,744	1,370 ± 90
+ NaN ₃	207,858 ± 1,587	1,256 ± 8
(OZ-stimulated macrophages)		
Unstimulated	18,734 ± 393*	200 ± 4*
Stimulated	343,403 ± 8,751	1,148 ± 21
+ H-7	192,125 ± 10,393*	623 ± 23*
+ W-7	221,934 ± 9,482*	456 ± 12*
+ HA-1004	333,017 ± 5,820	1,095 ± 34
+ W-5	328,107 ± 3,402	1,064 ± 33
+ NaN ₃	324,829 ± 2,541	1,098 ± 21

Experimental conditions are shown in **Materials and Methods**. Results are expressed as the mean ± SE for 3 samples. * $p < 0.001$, ** $p < 0.005$ when compared to the values when stimulated.

ation of O_2^- in TPA-stimulated MPs strongly depends on the activity of PKC and that the involvement of CaM could be neglected in the O_2^- generation in TPA-stimulated MPs. Furthermore, the results shown in Fig. 1c indicate that whether CaM or PKC is involved in the O_2^- generation in TPA-stimulated MPs cannot be decided by an O_2^- -unspecific luminol-dependent chemiluminescence method. From this point of view, the results shown in Fig. 1d are thought to be obtained accidentally and seemed not to indicate the truth.

By using the complicated and sample-wasting ferricytochrome *c* reduction method, it is widely accepted that O_2^- generation in TPA-stimulated PMNs depends on PKC.¹⁷⁾ We clearly demonstrated here the same phenomenon in TPA-stimulated MPs using a simple MCLA-dependent chemiluminescence method. On the other hand, in the investiga-

tions of OZ-stimulated PMNs, only CaM is reported to be involved.^{3,4,18,19)} However, we showed, using the MCLA-dependent chemiluminescence method, that not only CaM but also PKC are involved in the generation of O_2^- in OZ-stimulated MPs.

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