Enhancement of Zymosan-Induced Respiratory Burst of Rat Neutrophils by Lead *in Vitro*

Jun YATSUYANAGI,* Sanae MURAOKA, Ryou TSUTSUI and Taketo OGISO

Hokkaido Institute of Pharmaceutical Sciences, 7–1 Katsuraoka-chou, Otaru 047–02, Japan. *Received October 24, 1988*

The effect of Pb^{2+} on serum-treated zymosan (STZ)-induced O_2 consumption of rat peritoneal neutrophils was studied. Pb^{2+} was found to mimic effectively the enhancing action of Ca^{2+} on the O_2 consumption depending on the concentration up to about 80 μM. However, at concentrations over 80 μM, Pb^{2+} inhibited the O_2 consumption. The enhancing effect of Pb^{2+} on the O_2 consumption was further examined using the intracellularly Ca^{2+}-depleted neutrophils. Pb^{2+} also enhanced the O_2 consumption of the Ca^{2+}-depleted cells as effectively as Ca^{2+}. The Pb^{2+}-enhanced O_2 consumption of the Ca^{2+}-depleted cells was inhibited by *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) based on its calmodulin antagonistic action. The effect of Pb^{2+} on the activity of activator-deficient 3′,5′-cyclic nucleotide phosphodiesterase (PDE), a calmodulin-dependent enzyme, was examined. Pb^{2+} activated PDE as effectively as Ca^{2+} only in the presence of calmodulin. The Pb^{2+}-activated PDE activity was also inhibited by W-7 only in the presence of calmodulin. These results indicated that Pb^{2+} could replace Ca^{2+} in the activation process(es) of the respiratory burst, suggesting a possible involvement of calmodulin in the enhancing mechanism of the O_2 consumption by Pb^{2+}.

Keywords neutrophil; serum-treated zymosan; O_2 consumption; intracellularly Ca^{2+}-depleted neutrophil; lead; calcium; calmodulin; phosphodiesterase; W-7; W-5

It has been reported that lead induces changes in calcium homeostasis.[1] Calcium itself also has been shown to influence the absorption and distribution of lead.[2] At the cellular level, lead has been shown to affect the calcium-mediated α-adrenergic regulation of pyruvate kinase activity in isolated rat hepatocytes, suggesting that lead may alter the function of calcium as a second messenger.[3] On the other hand, it was demonstrated that potassium release from human red blood cells treated with calcium ionophore A-23187 was markedly increased by lead.[4] The potassium release from A-23187-treated red cells is known to depend on calmodulin.[4] In fact, it has been suggested on the basis of *in vitro* studies that lead binds to the calmodulin molecule leading to a conformational change similar to that caused by binding with calcium.[5] Thus, the possibility arises that lead affects the cell functions by substituting for or displacing calcium in regulatory processes involving calmodulin function. However, the precise role of calmodulin in lead toxicity has not been clarified yet.

In the course of our study concerning the effect of heavy metal ions on the functions of neutrophils, we found *in vitro* that either lead or calcium could enhance the respiratory burst of rat neutrophils. Previous workers have demonstrated that calmodulin is involved in the expression of various neutrophil functions, including the respiratory burst and degranulation process. In this report, we show that lead has an ability to enhance serum-treated zymosan (STZ)-induced O_2 consumption of rat neutrophils as a substitute for Ca^{2+} and we present evidence for possible involvement of calmodulin in the enhancing mechanism of the respiratory burst by lead.

Experimental

Materials Zymosan A, activator-deficient 3′,5′-cyclic nucleotide phosphodiesterase (from bovine heart) (PDE), *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), trifluoperazine (TFP), snake venom (from *Crotalus atrox*) and adenosine 3′,5′-cyclic monophosphate (cAMP) were obtained from Sigma Co., calmodulin (CaM) from Amano Seiyaku Co., β-nicotinamide adenine dinucleotide (NAD) from Oriental Yeast Co., ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) from Dojin Kagaku Kenkyusyo. $PbCl_2$ from Kanto Kagaku Co., and ionophore A-23187 from Calbiochem. All other reagents were of analytical grade.

Preparation of Neutrophil Suspension Neutrophil suspension was prepared according to the method described by Ichikawa and Imanishi[6] with modifications as follows. Neutrophils were obtained from peritoneal exudate of male Wistar rats (200—250 g) 15 h after the peritoneal injection of 8 ml of 8% sodium caseinate solution. The peritoneal cells were centrifuged (170 × *g*, 10 min) and washed in normal saline. Residual erythrocytes were eliminated by hypotonic lysis. The cell fractions obtained by this procedure contained more than 85% neutrophils with more than 95% viability as determined by the trypan blue dye exclusion test. The major contaminating cell type was mononuclear cells. The cells were resuspended in Hepes-buffered saline containing 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM glucose and 17 mM Hepes, pH 7.4 (HBS medium), to make 2×10^7 cells/ml.

Measurement of O_2 Consumption The decrease of O_2 concentration in the cell suspension was measured as described previously[7] by using a Clark-type oxygen electrode (type 5331, Yellow Springs Instrument Co., OH, U.S.A.) fitted in a closed vessel (0.6 ml) kept at 37 °C with magnetic stirring. Cell suspension (0.5 ml, 2×10^7 cells/ml of HBS medium containing 50 μM EGTA) was added to the vessel and incubated for 10 min at 37 °C. The reaction was started by the addition of STZ (2.5 mg/10^7 cells) and the change of O_2 concentration was recorded. The electrode was calibrated according to the method described by Friedovich and Misra.[8]

Preparation of STZ Zymosan was incubated in fresh rat serum to be opsonized according to the method described previously.[9]

Preparation of Intracellularly Ca^{2+}-Depleted Neutrophils Neutrophils exhaustively deprived of Ca^{2+} were prepared according to the method described by Minakami *et al.*[10] as follows. The cells (1×10^7/ml) were incubated in a divalent cation-free HBS medium with 1 μM ionophore A-23187 and 0.1 mM EGTA for 10 min at 37 °C, washed twice and then resuspended in cold HBS medium at the final concentration of 2×10^7 cells/ml. Viability of the depleted cells was more than 95%, as assessed by the trypan blue dye exclusion test.

Assay of PDE Activity Activity of PDE was assayed by the method of Butcher and Sutherland[11] with minor modifications as follows. The reaction mixture contained 0.36 mM cAMP, 1.8 mM $MgSO_4$, 35 μg protein of PDE, 5 μg of CaM unless otherwise stated, 50 μM EGTA and 100 μM Ca^{2+} or various concentrations of Pb^{2+} in 1 ml of 240 mM Tris buffer, pH 7.5. The reaction mixture was incubated for 30 min at 30 °C, and then the reaction was stopped by heating in a boiling water bath for 1 min. After cooling, 0.1 ml of *Crotalus atrox* venom solution containing 0.1 mg of the venom in 10 mM Tris buffer, pH 7.5, was added and the mixture was incubated for 10 min at 30 °C. Ethylenediaminetetraacetic acid (EDTA) (200 μM) was included to prevent the formation of a precipitate in the experiments using Pb^{2+}. The reaction was terminated by the addition of 0.1 ml of 55% trichloroacetic acid and the precipitate was removed by centrifugation. The amount of inorganic phosphate in the supernatant was measured by the method of Fiske and SubbaRow.[12]

Assay of Lactate Dehydrogenase (LDH) Activity Activity of LDH was assayed by measuring the conversion of NAD to reduced nicotinamide adenine dinucleotide (NADH) during the reaction of lactate to pyruvate as described previously.[9]

Protein Determination The protein concentration was determined with BCA protein assay reagent (Pierce Chemical Co., U.S.A.), using bovine serum albumin as a standard as described previously.[7]

Results

Effect of Pb^{2+} on STZ-Induced O_2 Consumption of Neutrophils As shown in Fig. 1, addition of Pb^{2+} to the incubation medium resulted in an enhancement of STZ-induced O_2 consumption by neutrophils depending on the concentration of Pb^{2+}, up to about 80 μM. The rate of the O_2 consumption was about 21.5 nmol/min/10^7 cells at 80 μM Pb^{2+}. However, Pb^{2+} inhibited the O_2 consumption of neutrophils at concentrations above 100 μM. Ca^{2+} also enhanced the O_2 consumption depending on the concentration up to about 100 μM, reaching a plateau at concentrations above 100 μM. These results indicate that

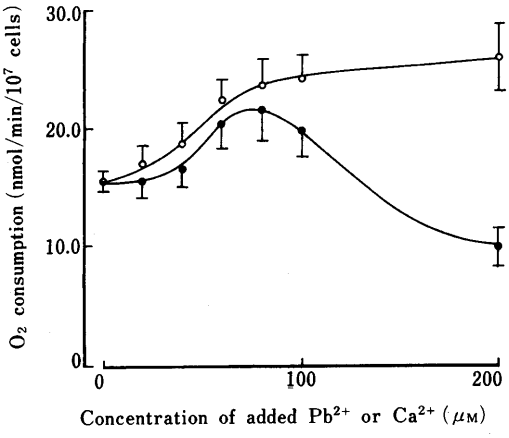

Fig. 1. Effect of Pb^{2+} or Ca^{2+} on STZ-Induced O_2 Consumption of Neutrophils

Neutrophils were suspended in HBS medium containing 50 μM EGTA and various amounts of Ca^{2+} (○) or Pb^{2+} (●), and incubated for 10 min at 37 °C. Consumption of O_2 was induced by the addition of STZ (2.5 mg/10^7 cells). Each point represents the mean ± S.E. of 3 measurements.

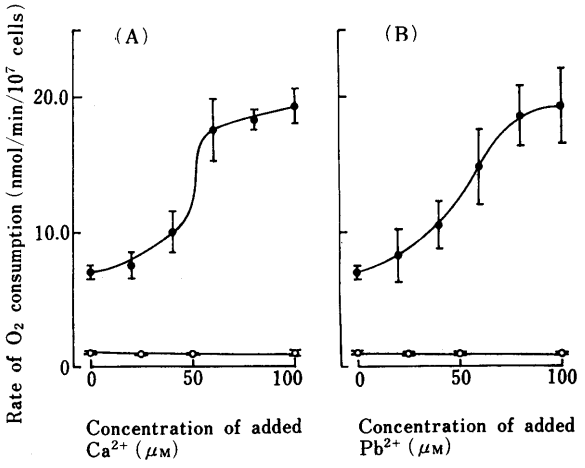

Fig. 2. Effect of Pb^{2+} or Ca^{2+} on O_2 Consumption of Ca^{2+}-Depleted Neutrophils

The Ca^{2+}-depleted neutrophils were stimulated with STZ (2.5 mg/10^7 cells), after preincubation for 10 min at 37 °C in HBS medium containing various amounts of Ca^{2+} or Pb^{2+} in the presence of 50 μM EGTA (●). Each point represents the mean ± S.E. of 3 measurements. (○): O_2 consumption of Ca^{2+}-depleted cells in the resting state in the presence of various amounts of Ca^{2+} or Pb^{2+}

Pb^{2+} could enhance STZ-induced O_2 consumption as effectively as Ca^{2+}, but its effect was biphasic, *i.e.*, a relatively high concentration of Pb^{2+} caused an inhibition of the O_2 consumption.

Enhancement of O_2 Consumption of Ca^{2+}-Depleted Neutrophils by Pb^{2+} The enhancing effect of Pb^{2+} on the O_2 consumption of neutrophils was examined by using intracellularly Ca^{2+}-depleted neutrophils. The O_2 consumption of the Ca^{2+}-depleted cells induced with STZ was about 7.0 nmol/min/10^7 cells. As can be seen in Fig. 2A, the O_2 consumption was enhanced by the addition of Ca^{2+} depending on the concentration and reached almost the same level as that of normal cells (19.3 nmol/min/10^7 cells). Cell viability of the Ca^{2+}-depleted cells, assessed by the trypan blue dye exclusion test, was not significantly changed from that of normal cells. As shown in Fig. 2B, on the other hand, Pb^{2+} could enhance the O_2 consumption of the depleted cells to a level comparable to that observed in the presence of 100 μM Ca^{2+}. Neither Pb^{2+} nor Ca^{2+} significantly affected the O_2 consumption of the resting cells. These results indicate that Pb^{2+} can also enhance the O_2 consumption of Ca^{2+}-depleted neutrophils as a substitute for Ca^{2+}.

Inhibition of Pb^{2+}- or Ca^{2+}-Enhanced O_2 Consumption of Ca^{2+}-Depleted Neutrophils by W-7 and W-5 Pb^{2+} has been shown *in vitro* to interact with calmodulin causing a conformational change comparable to that caused by the interaction with Ca^{2+}.[5c] Therefore, we examined the effect of calmodulin inhibitors W-7 and W-5 (a chlorine-deficient analogue of W-7[13]) on Pb^{2+}-enhanced O_2 consumption of the Ca^{2+}-depleted neutrophils. As shown in Table I, the O_2 consumption of the Ca^{2+}-depleted cells induced with STZ in the presence of 100 μM Pb^{2+} was markedly inhibited by W-7 in a concentration-dependent manner. The extent of inhibition caused by 10, 25 or 50 μM W-7 were about 25%, 64% or 91%, respectively, while those by 25 or 50 μM W-5 were about 27% or 46%, respectively. On the other hand, the inhibitory actions of W-7 and W-5 on the O_2 consumption of Ca^{2+}-depleted cells in the presence of 100 μM Ca^{2+} were about the same as those in the presence of 100 μM Pb^{2+}. Neither W-7 nor W-5 caused any significant cell damage at the concentrations employed in this experiment, as assessed in terms of the leakage of LDH, in the presence of 100 μM Pb^{2+}, indicating that the

TABLE I. Inhibition of Pb^{2+}- or Ca^{2+}-Activated O_2 Consumption by W-7 and W-5 in Ca^{2+}-Depleted Neutrophils

	Rate of O_2 consumption (nmol/min/10^7 cells)			
	Pb^{2+} (100 μM)	Inhibition %	Ca^{2+} (100 μM)	Inhibition %
Control	17.4 ± 3.5		17.8 ± 3.0	
W-7 (μM)				
10	13.1 ± 3.0	25.0	13.7 ± 2.9	25.0
25	6.5 ± 1.8	64.2	8.3 ± 1.8	54.4
50	2.0 ± 0.7	91.1	3.7 ± 0.9	80.2
W-5 (μM)				
25	13.3 ± 3.4	26.7	15.0 ± 2.9	17.1
50	7.4 ± 1.5	46.2	12.1 ± 2.5	32.9

The Ca^{2+}-depleted neutrophils were incubated in HBS medium containing 100 μM Pb^{2+} or 100 μM Ca^{2+} for 10 min at 37 °C in the presence of various amounts of W-7 or W-5, and then stimulated by the addition of STZ (2.5 mg/10^7 cells). Each value represents the mean ± S.E. of 3 measurements.

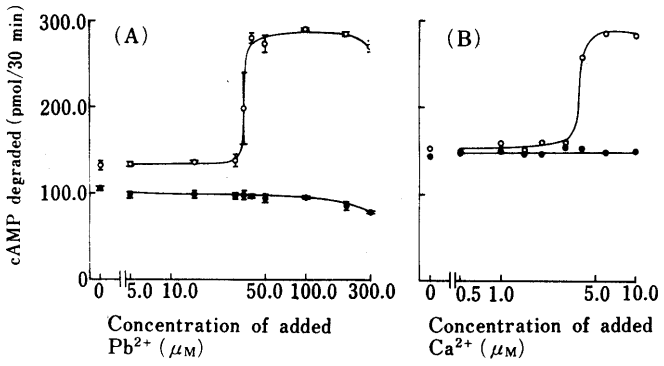

Fig. 3. Activation of PDE by Pb^{2+} or Ca^{2+}

Activities of PDE were assayed in the presence of various amounts of Pb^{2+} (A) or Ca^{2+} (B) with 1 μg CaM (○) or without CaM (●). Each point in panel A represents the mean ± S.E. of 3 measurements. Panel B shows representative results.

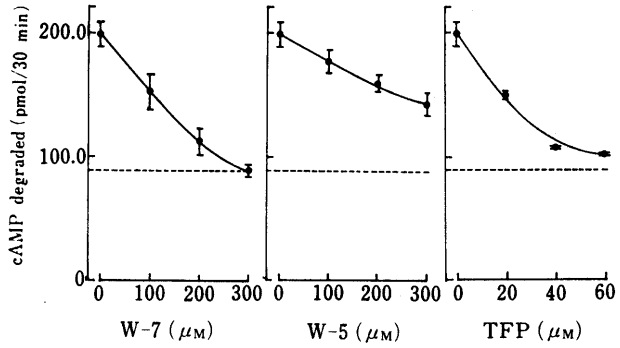

Fig. 4. Inhibition of Pb^{2+}-Activated PDE by Calmodulin Inhibitors

Activities of PDE were measured in the presence of Pb^{2+} (100 μM). CaM (0.25 μg) and various amounts of calmodulin inhibitors. The dotted line indicates the basal activity of PDE observed in the absence of calmodulin. Each point represents the mean ± S.E. of 3 measurements.

observed inhibitory action of W-7 or W-5 is not due to cytotoxic action. In addition, the fact that the inhibitory action of W-5 is less pronounced than that of W-7 indicates that the inhibitory action of W-7 is due to its calmodulin-antagonistic action, suggesting a possible participation of calmodulin in the enhancing mechanism of STZ-induced O_2 consumption by Pb^{2+}.

Activation of PDE by Pb^{2+} Further, to ascertain whether Pb^{2+} can activate calmodulin-dependent enzymes, we selected PDE, which is known to be a calmodulin-stimulated enzyme, and examined the effect of Pb^{2+} on its activity. As demonstrated in Fig. 3A, PDE activity was enhanced by 50 μM Pb^{2+} to 272.9 from the basal level of 131.9 pmol cAMP degraded/30 min. The activity of PDE at 100 μM Pb^{2+} was 290.5 pmol cAMP degraded/30 min, comparable to that observed in the presence of 10 μM Ca^{2+} (Fig. 2B). The activation of PDE by Pb^{2+} or Ca^{2+} was observed only in the presence of calmodulin. The PDE activity was slightly decreased by 300 μM Pb^{2+} in the presence or absence of calmodulin, indicating that PDE was inhibited at a relatively high concentration of Pb^{2+}.

As can be seen in Fig. 4, Pb^{2+}-activated PDE activity was inhibited only in the presence of calmodulin by W-7 in a concentration dependent fashion and was decreased to the basal level by 300 μM W-7. On the other hand, the inhibitory action of W-5 was less pronounced than that of W-7. The activity was also inhibited by trifluoperazine (TFP), another type of calmodulin inhibitor, in a concentration dependent manner. These results indicate that Pb^{2+}–calmodulin complex has an ability to activate PDE, a calmodulin-dependent enzyme, as a substitute for Ca^{2+}–calmodulin complex.

Discussion

Activation of neutrophils is characterized by a marked increase of cyanide-insensitive O_2 consumption. This respiratory burst results in the production of large quantities of reactive oxygen metabolites such as O_2^- and H_2O_2.[14] The present study demonstrated that Pb^{2+} could enhance the STZ-induced respiratory burst of neutrophils at low concentrations (Fig. 1). It has been established that the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased by stimulating neutrophils with receptor-mediated triggers such as *n*-formyl-metionyl-leucyl-phenylalanine (FMLP) and STZ, resulting in the activation of O_2 consumption,[15] and that calmodulin is one of the target proteins for Ca^{2+} binding. Two types of mechanisms are regarded as being involved in the trigger-induced increase of $[Ca^{2+}]_i$: one is the increase of Ca^{2+} influx from the extracellular medium through the cell membrane and the other is the release of Ca^{2+} from intracellular Ca^{2+} storage sites.[16] Therefore, we examined the effect of Pb^{2+} on the O_2 consumption in neutrophils deprived of Ca^{2+} by treatment of the cells with ionophore A-23187 in the presence of EGTA. The O_2 consumption of the Ca^{2+}-depleted cells stimulated with STZ was markedly enhanced by the addition of Ca^{2+}, reaching the almost same level as that of normal cells, indicating that the Ca^{2+}-depleted cells predominantly utilize extracellular Ca^{2+} in the activation mechanism of O_2 consumption (Fig. 2A). Addition of Pb^{2+} also enhanced the O_2 consumption of the Ca^{2+}-depleted cells, suggesting that Pb^{2+} can substitute for Ca^{2+} in the activation mechanism of the O_2 consumption (Fig. 2B). Other workers have demonstrated by using calmodulin inhibitors such as W-7 or TFP that calmodulin plays a role in the Ca^{2+}-mediated activation of the phagocytic respiratory burst and degranulation of neutrophils.[17] In the present study, the enhanced O_2 consumption by Pb^{2+} or Ca^{2+} was markedly inhibited by W-7 based on its calmodulin antagonistic action (Table I), indicating an involvement of calmodulin in the enhancing mechanism of the O_2 consumption by Pb^{2+}.

Calmodulin-dependent PDE has been reported to be activated with Ca^{2+}, Pb^{2+}, Sr^{2+}, Ba^{2+} and Cd^{2+}.[5a] Our results also demonstrated that Pb^{2+} could activate PDE only in the presence of calmodulin and that Pb^{2+} could substitute for Ca^{2+} to activate PDE (Fig. 3). Moreover, our finding that Pb^{2+}-activated PDE was inhibited by W-7 or TFP only in the presence of calmodulin suggest an interaction of Pb^{2+} with calmodulin in the activation process of PDE (Fig. 4). These results were further supported by the results of previous workers[5c] that the binding of Ca^{2+} or Pb^{2+} to calmodulin induced an altered conformation with an increased quantum yield in its tyrosine fluorescence. These facts suggest that Pb^{2+} causes an conformational change of calmodulin similar to that evoked by binding of Ca^{2+}. Although calmodulin has been postulated to be involved in the Ca^{2+}-mediated activation of the phagocytic respiratory burst, the target enzyme of activated calmodulin in neutrophils has not been elucidated. Takeshige and Minakami have demonstrated that NADPH oxidase pre-

pared from cytochalasin D-stimulated neutrophils was inhibited by TFP.[17b] In addition, McCord *et al.* reported that the activity of reduced nicotinamide adenine diphosphate (NADPH) oxidase prepared from zymosan-stimulated neutrophils was enhanced by exogenous calmodulin.[18] These facts suggest that NADPH-oxidase may be one of the calmodulin-regulated enzyme in neutrophils. However, further study is needed to clarify the possible role of calmodulin in the activation process(es) of NADPH oxidase.

Earlier workers have demonstrated that Pb^{2+} affects the Ca^{2+}-mediated cell functions, including the regulation of pyruvate kinase by glucagon or epinephrine in the hepatocytes,[1b] the promotion of potassium loss from erythrocytes,[4] and the inhibition of synaptic transmission in sympathetic ganglia.[19] From these results, calmodulin has been suggested to be a mediator of some of the toxic effects of Pb^{2+}.[5c] Nevertheless, the present finding that the effect of Pb^{2+} on the respiratory burst in neutrophils is biphasic (stimulatory at low concentrations and inhibitory at high concentrations) does not necessarily mean that Pb^{2+} exerts its toxicity through the activation of calmodulin. Actually, concentrations of Pb^{2+} greater than 80 μM caused a marked inhibition of $[Ca^{2+}]_i$ increase associated with neutrophil activation (data not shown). Further studies should be done to elucidate the cellular events involved in the pathogenesis of Pb^{2+} toxicity.

References

1) *a*) K. M. Six and R. A. Goyer, *J. Lab. Clin. Med.*, **76**, 933 (1970); *b*) K. R. Mahaffey, R. Goyer and J. K. Haseman, *ibid.*, **82**, 92 (1973); *c*) J. G. Pounds, R. Wright, D. Morrison and D. A. Casciano, *Toxicol. Appl. Pharmacol.*, **63**, 389 (1982): *d*) J. G. Pounds, *Neurotoxicology*, **5**, 295 (1984).
2) *a*) N. Gruden, M. Stantic and M. Buden, *Environ. Res.*, **8**, 203 (1974); *b*) N. Gruden, *Toxicology*, **5**, 163 (1975); *c*) N. Gruden and M. Buden, *Bull. Environ. Contam. Toxicol.*, **18**, 303 (1977).
3) J. G. Pound, D. Morrison. R. Wright, D. A. Casciano and J. G. Shaddock, *Toxicol. Appl. Pharmacol.*, **63**, 402 (1982).
4) G. W. Goldstein and D. Ar, *Life Sci.*, **33**, 1001 (1983).
5) *a*) E. Haberman, K. Crowell and P. Janicki, *Arch. Toxicol.*, **54**, 61 (1983); *b*) S. H. Chao, Y. Suzuki, J. R. Zysk and W. Y. Cheung, *Mol. Pharmacol.*, **26**, 75 (1984); *c*) G. Richardt, G. Federolf and E. Habermann, *Biochem. Pharmacol.*, **35**, 1331 (1986).
6) "Modern Application of Cultured Animal Cells in Experimental Medicine," ed. by H. Endou, A. Ichikawa and N. Imanishi, R&D Planning, Tokyo, 1985, Chapter II, Section 4—6, p. 130, (in Japanese).
7) J. Yatsuyanagi and T. Ogiso, *Chem. Pharm. Bull.*, **36**, 1035 (1988).
8) I. Friedovich and H. P. Misra, *Anal. Biochem.*, **70**, 632 (1976).
9) J. Yatsuyanagi, K. Iwai and T. Ogiso, *Chem. Pharm. Bull.*, **35**, 699 (1987).
10) M. Nakagawara, K. Takesige, H. Sumimoto, J. Yoshitake and S. Minakami, *Biochim. Biophys. Acta*, **805**, 97 (1984).
11) R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.*, **237**, 1244 (1962).
12) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).
13) H. Hidaka, Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii and T. Nagata, *Proc. Nat. Acad. Sci. U.S.A.*, **78**, 4354 (1981).
14) J. A. Badwey and M. L. Karnovsky, *Ann. Rev. Biochem.*, **49**, 695 (1980).
15) F. Rossi, V. D. Bianca, M. Grzeskowiak, P. De Togni and G. Cabrini, *FEBS Lett.*, **181**, 253 (1985).
16) T. Anderson, C. Dahlgren, T. Pozzan, O. Stendahl and P. D. Lew, *Mol. Pharmacol.*, **30**, 437 (1986).
17) *a*) P. H. Naccache, T. F. P. Molski, T. Alobaidi, E. L. Becker, H. J. Showell and R. I. Sha'afi, *Biochem. Biophys. Res. Commun.*, **97**, 62 (1980); *b*) K. Takeshige and S. Minakami, *ibid.*, **99**, 484 (1981); *c*) C. D. Wright and M. D. Hoffman, *ibid.*, **142**, 53 (1987).
18) H. P. Jones, G. Ghai, W. F. Petrone and J. M. McCord, *Biochim. Biophys. Acta*, **714**, 152 (1982).
19) T. E. Kober and G. P. Cooper, *Nature* (London), **262**, 704 (1976).