Volume 221, number 1, 23-27 FEB 05044 August 1987

Synthetic diacylglycerols induce a rise of quin2-detectable free intracellular calcium in human platelets

Christian Bruns*, Christoph Schächtele and Dieter Marmé

Gödecke Research Institute, Mooswaldallee 1-9, 7800 Freiburg, FRG

Received 15 June 1987; revised version received 10 July 1987

The two activators of protein kinase C, oleoylacetylglycerol (OAG) and dioctanoylglycerol (DOG), are able to induce a concentration-dependent rise in cytoplasmic free Ca^{2+} concentration in gel-filtered human platelets, detected as an increase in quin2 fluorescence. The phorbol ester phorbol-12-myristate-13-acetate (PMA) has no effect. The OAG-induced increase of intracellular Ca^{2+} is not influenced by forskolin, in contrast to the effect of the diterpene on the thrombin-stimulated increase in cytoplasmic Ca^{2+}. It is concluded that the increase in intracellular free Ca^{2+} concentration induced by synthetic diacylglycerols and their activation of protein kinase C are two different and independent processes.

Diacylglycerol; Protein kinase C; Quin2; Forskolin; Ca^{2+}; (Human platelet)

1. INTRODUCTION

Stimulation of platelets by various neurotransmitters, hormones, and other biologically active substances results in the activation of two enzymes, i.e. myosin light chain kinase and protein kinase C [1-3]. Activation of these enzymes is often preceded by receptor-mediated hydrolysis of phosphoinositides which generates diacylglycerol, the physiological activator of PKC, and inositol 1,4,5-trisphosphate which releases Ca^{2+} from intracellular compartments [4-7]. Whereas activation of myosin light chain kinase requires an increase in free cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$, as a prerequisite [8], PKC stimulation is assumed to occur also at resting calcium [9,10]. PKC and myosin light chain kinase once activated lead in a synergistic mode of action to secretion of dense body constituents, such as serotonin and ADP, and aggregation [1,3,11].

Under in vitro conditions, activation of PKC can be achieved using various compounds [12-14], such as OAG, DOG and PMA. Furthermore, it could be shown that stimulation of platelets by these agents results in aggregation [9,15,16], but with none of these stimuli could an increase in $[Ca^{2+}]_i$ in platelets be demonstrated when the quin2 method was used for calcium detection [9,10,17]. In contrast, elevation of $[Ca^{2+}]_i$ was observed by means of the photoprotein aequorin after stimulation of platelets with PMA and OAG [17].

Recently is has been reported that incubation of saponin-permeabilized human platelets with OAG resulted in release of $^{45}Ca^{2+}$ from the dense-tubular system [18]. Since the time course of OAG-induced $^{45}Ca^{2+}$ release corresponded to that of conversion of OAG to OAG-phosphatidic acid, the authors suggest that the $^{45}Ca^{2+}$ release caused

Correspondence address: C. Schächtele, Gödecke Research Institute, Mooswaldallee 1-9, 7800 Freiburg, FRG

* Present address: Preclinical Research Department, Sandoz Ltd, Basel, Switzerland

Abbreviations: DOG, 1,2-dioctanoyl-*sn*-glycerol; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; PKC, protein kinase C; PMA, 4β-phorbol-12-myristate-13-acetate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

by diacylglycerol is due to the formation of phosphatidic acid. In contrast, no increase of $^{45}Ca^{2+}$ could be detected upon platelet stimulation with PMA [18].

Based on these different results concerning the $[Ca^{2+}]_i$ increase upon platelet stimulation with various PKC activators, we reexamined the question as to whether an increase in $[Ca^{2+}]_i$ after platelet stimulation with OAG and DOG could be observed using the intracellular fluorescent probe quin2. We were able to show that OAG and DOG when added to gel-filtered human plates caused a concentration-dependent rise of $[Ca^{2+}]_i$ whereas no effect could be observed with PMA.

2. MATERIALS AND METHODS

Quin2 acetoxymethyl ester (quin2-AM), OAG, PMA, thrombin and forskolin were purchased from Sigma (München), DOG was synthesised in the Department of Chemical Research, Gödecke (Freiburg). Sepharose Cl-2B was from Pharmacia (Freiburg). All other chemicals used were of analytical grade and obtained from commercial sources.

2.1. *Quin2 measurement*

Intracellular free calcium concentrations in human platelets were determined using the fluorescent indicator quin2 as described by Tsien et al. [19].

Citrated blood was obtained from healthy volunteers by venous puncture and immediately centrifuged at 60 × *g* for 15 min to obtain platelet-rich plasma. The platelet-rich plasma was incubated with 20 μM quin2-AM [20] for 30 min at 37°C. Subsequently the quin2-loaded platelets were further purified as described by Tangen et al. [21] using a Sepharose Cl-2B column equilibrated at room temperature with 15 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5.5 mM glucose and 0.2% bovine serum albumin. After pooling the peak fractions, the platelet concentration was determined using a Coulter counter (Coulter Electronics, Krefeld, FRG). The final platelet concentration was adjusted to 200000 platelets/μl. The quin2-loaded platelets were supplemented with 0.7 mM Ca^{2+} for 15 min at room temperature. Drugs were added to the stirred platelet suspension followed by fluorescence recording with a spectrofluorimeter (Perkin-Elmer MPF-2A). DMSO at a final concentration of 1% was used as vehicle, which by itself did not effect quin2-detectable $[Ca^{2+}]_i$.

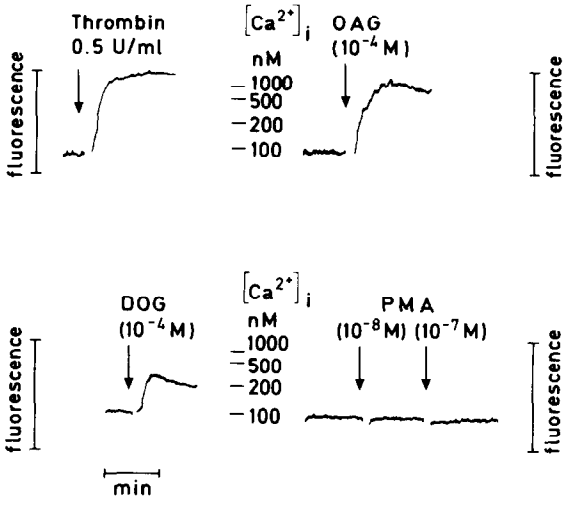

Fig.1. Stimulus-dependent increase in $[Ca^{2+}]_i$ in gel-filtered human platelets. Original traces of a representative experiment are presented. Detailed experimental conditions are described in section 2.

In order to calculate the intracellular free calcium concentration, loaded platelets were lysed at 37°C with 0.02% Triton X-100; maximal fluorescence was measured after adding 3 mM $CaCl_2$, minimal fluorescence after adding 10 mM

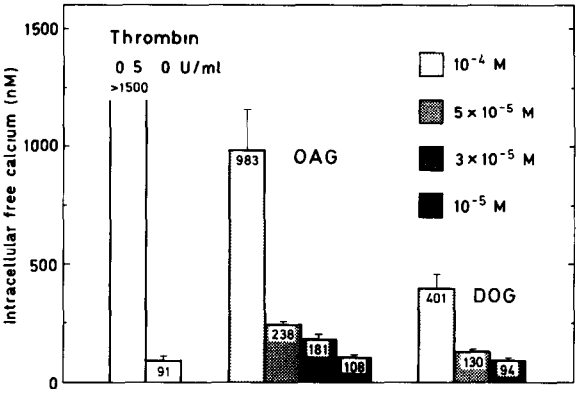

Fig.2. Increase of $[Ca^{2+}]_i$ induced by various concentrations of OAG and DOG. Values are expressed as means ± SE of $n = 40$ (basal), $n = 5$ (OAG) and $n = 5$ (DOG) individual experiments.

Table 1

Effect of EGTA on basal and diacylglycerol-induced rise of $[Ca^{2+}]_i$ in gel-filtered human platelets

Stimulus	$[Ca^{2+}]_i$ (nM)		n
	At 0.7 mM Ca^{2+}	At 3 mM EGTA	
None	91 ± 2	76 ± 5	40/11
OAG (10^{-4} M)	983 ± 17	319 ± 39	5
DOG (10^{-4} M)	401 ± 7	257 ± 23	5

3 mM EGTA was added to gel-filtered, quin2-loaded platelets 2 min before stimulation with OAG or DOG. Data are means ± SE of the indicated number of individual experiments

EGTA and raising the pH to 8.3. Calibration of intracellular fluorescence as a function of $[Ca^{2+}]_i$ was performed as in [19,22].

3. RESULTS

Using the quin2 method, the resting level of $[Ca^{2+}]_i$ in unstimulated gel-filtered human platelets was determined to be 91 ± 6 nM ($n = 40$). As shown in fig.1, the addition of OAG (10^{-4} M) and DOG (10^{-4} M) resulted in a rapid increase in $[Ca^{2+}]_i$ within 30 s. This rise in $[Ca^{2}]_i$ was transient and decreased within a few minutes (not shown). In contrast, we could not detect an increase of $[Ca^{2+}]_i$ when platelets were stimulated with PMA. Even at concentrations as high as 10^{-7} M, PMA did not alter the resting calcium level as indicated by the unchanged quin2 fluoresence (fig.1). For comparison the effect of the physiological stimulus thrombin on intracellular free Ca^{2+} is also shown in fig.1. As depicted in fig.2 the rise of $[Ca^{2+}]_i$ in response to OAG and DOG stimulation is concentration-dependent. It should be noted that OAG is slightly more effective than DOG.

In the presence of 3 mM EGTA, OAG and DOG were still able to cause an increase in $[Ca^{2+}]_i$ (table 1). This indicates that an internally releasable calcium pool contributes to the rise of $[Ca^{2+}]_i$.

To characterize further the observed Ca^{2+} increase we investigated the effects of forskolin on

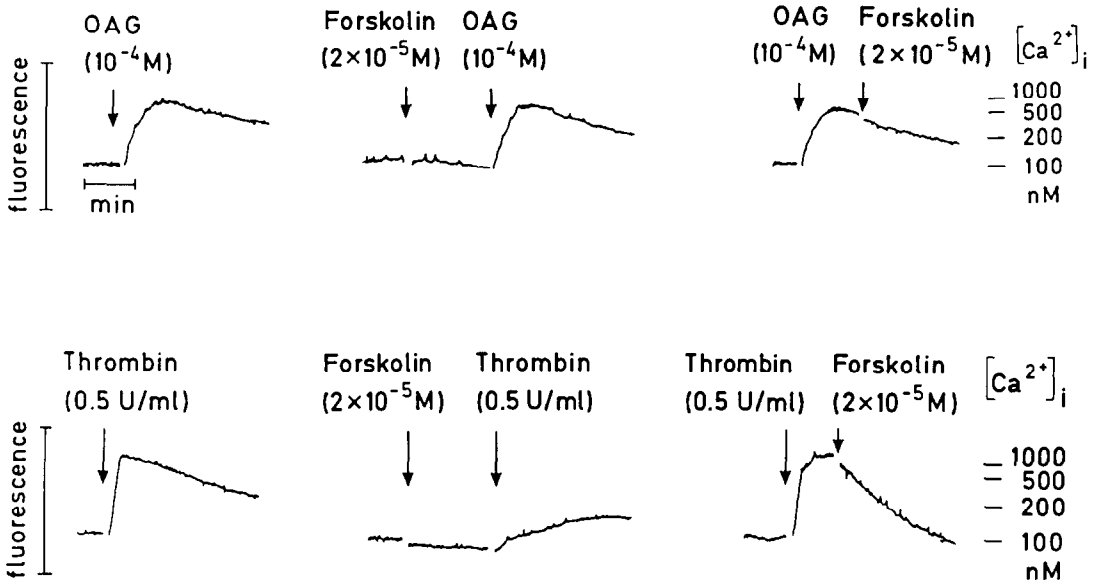

Fig.3. Effect of forskolin on the OAG-induced rise of $[Ca^{2+}]_i$ (upper panel). For comparison, the effect of forskolin on the thrombin-induced increase in $[Ca^{2+}]_i$ is shown in the lower panel.

the OAG-induced rise of $[Ca^{2+}]_i$. The amplitude or time course of the Ca^{2+} signal was changed by neither preincubation with forskolin nor addition at maximal $[Ca^{2+}]_i$ (fig.3, upper panel). This contrasts with the well-documented inhibitory effect of forskolin on the thrombin-induced rise of $[Ca^{2+}]_i$ ([23–25] and fig.3, lower panel).

4. DISCUSSION

Synthetic diacylglycerols like OAG, as well as phorbol esters such as PMA, have been reported to induce platelet aggregation without a concomitant rise of $[Ca^{2+}]_i$ when quin2 fluorescence is used to probe for free cytoplasmic Ca^{2+} [3,9,17]. This led to the assumption that OAG, DOG and PMA were able to induce this response at resting $[Ca^{2+}]_i$ and that the synergistic activation by diacylglycerol and Ca^{2+} is not a conditio sine qua non for platelet aggregation. However, in two recent publications using different techniques data were presented which indicated a rise of $[Ca^{2+}]_i$ upon platelet stimulation with OAG. In one case, aequorin was loaded into platelets and subsequent addition of OAG caused a considerable increase of luminescence [17]. The authors of the second paper used saponin-permeabilized platelets loaded with $^{45}Ca^{2+}$. They were able to show that OAG caused a release of $^{45}Ca^{2+}$ presumably from the dense-tubular system [18]. These data suggest that the negative results obtained with the quin2 method could have been due to inappropriate experimental conditions.

We therefore decided to reexamine the effects of OAG, DOG and PMA on free Ca^{2+} levels in gel-filtered human platelets by using the quin2 method. We were able to demonstrate that both OAG and DOG could induce an increase in $[Ca^{2+}]_i$. This increase was concentration-dependent and corresponds to those results obtained with aequorin and $^{45}Ca^{2+}$ [17,18]. Considering the results obtained with the $^{45}Ca^{2+}$ method by Brass and Laposata [18], it is likely that the rise of $[Ca^{2+}]_i$ as dectected with the quin2 method is not due to a direct effect of OAG but, rather, implies the conversion of OAG to OAG-phosphate. This phosphatidic acid in turn releases Ca^{2+} from intracellular compartments. This view is supported by the observation that in HL-60 cells the addition of synthetic diacylglycerols causes a decrease of cellular ATP [27].

It is well documented in the literature that forskolin, by increasing the cellular cAMP concentration, impairs the thrombin-induced rise of free cytoplasmic Ca^{2+} [23,25,26]. In contrast to these observations, we could not detect an effect of forskolin on the OAG-stimulated increase in $[Ca^{2+}]_i$. This indicates that the underlying mechanisms which control the thrombin- and OAG-dependent increase in $[Ca^{2+}]_i$ are different.

In agreement with data reported using the $^{45}Ca^{2+}$ method [18], we were not able to induce an increase in quin2 fluorescence with the phorbol ester PMA. This contrasts with the results obtained with aequorin which demonstrate a concentration-dependent increase of $[Ca^{2+}]_i$ by PMA [17]. The reason for this discrepancy is not understood at present and awaits further investigation.

From our data obtained with OAG and DOG one may conclude that stimulation of platelet aggregation by these synthetic diacylglycerols might not be due to the activation of the PKC alone but might also include a rise of free cytoplasmic Ca^{2+} concentration. However, a comparison of the concentration-response curves for OAG-induced platelet aggregation and the OAG-stimulated rise of $[Ca^{2+}]_i$ demonstrates that maximal platelet aggregation can be obtained at OAG concentrations (3×10^{-5} M) at which only a slight increase in free cytoplasmic Ca^{2+} was observed (Schächtele et al., in preparation). When added at higher concentrations, OAG induced both maximal aggregation and increase in $[Ca^{2+}]_i$.

Thus, in agreement with data obtained with the $^{45}Ca^{2+}$ and aequorin methods [17,18], we were able to demonstrate a quin2-detectable rise of $[Ca^{2+}]_i$ induced by the synthetic diacylglycerols OAG and DOG. The contrasting results obtained by others [3,9,10] might be explained by the fact that they did not use gel-filtered platelets. However, other differences in experimental procedure may also contribute to these discrepancies.

The physiological relevance of the OAG-mediated rise of $[Ca^{2+}]_i$ at least with respect to platelet aggregation awaits further clarification. However, our data indicate that the effects observed with synthetic diacylglycerols when used as experimental tools might not always be due to the selective activation of PKC but could also include

processes depending on the increase in free intracellular Ca^{2+}.

ACKNOWLEDGEMENTS

We would like to thank Mrs H. Schmitt and Mrs C. Trarbach for typing the manuscript and Mr K. Gössl for preparing the figures.

REFERENCES

[1] Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701-6704.
[2] Sano, K., Takai, Y., Yamanishi, J. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010-2013.
[3] Rink, T.J. and Hallam, T.J. (1984) Trends Biochem Sci. May, 215-219.
[4] Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. and Nishizuka, Y. (1982) Cell Calcium 3, 323-335.
[5] Brass, L.F. and Joseph, S.K. (1985) J. Biol. Chem. 260, 15172-15179.
[6] Adunyah, S.E. and Dean, W.L. (1985) Biochem. Biophys. Res. Commun. 128, 1274-1280.
[7] Siess, W. (1986) Biochem. Pharmacol. 35, 3184-3187.
[8] Hallam, T.J., Daniel, J.L., Kendrick-Jones, J. and Rink, T.J. (1985) Biochem. J. 232, 373-377.
[9] Rink, T.J., Sanchez, A. and Hallam, T.J. (1983) Nature 305, 317-319.
[10] Sanchez, A., Hallam, T.J. and Rink, T.J. (1983) FEBS Lett. 164, 43-46.
[11] Nishizuka, Y. (1984) Nature 308, 693-698.
[12] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
[13] Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y. and Fujikura, T. (1982) J. Biochem. 91, 427-431.
[14] Ebeling, J.G., Vandenbark, G.R., Kuhn, L.J., Ganong, B.R., Bell, R.M. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 815-819.
[15] Naka, M., Nishikawa, M., Adelstein, R.S. and Hidaka, H. (1983) Nature 306, 490-492.
[16] Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) J. Biol. Chem. 260, 1358-1361.
[17] Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W. (1985) Biochem. Biophys. Res. Commun. 133, 98-104.
[18] Brass, L.F. and Laposata, M. (1987) Biochem. Biophys. Res. Commun. 142, 7-14.
[19] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell. Biol. 94, 325-334.
[20] Erne, P., Mittelholzer, E., Bürgisser, E., Fluckiger, R. and Bühler, F.R. (1984) J. Receptor Res. 4, 587-604.
[21] Tangen, O., Berman, H.J. and Marfey, P. (1971) Thromb. Diath. Haemorrh. 25, 268-278.
[22] Rink, T.J., Smith, S.W. and Tsien, R.Y. (1982) FEBS Lett. 148, 21-26.
[23] Zavaico, G.B. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 120, 579-585.
[24] Feinstein, M.B., Halenda, S.P. and Zavoico, G.B. (1985) in: Calcium and Cell Physiology (Marmé, D. ed.) pp. 345-376, Springer, Berlin.
[25] Sage, S.O. and Rink, T.J. (1985) FEBS Lett. 188, 134-140.
[26] Yamanishi, J., Kawahara, Y. and Fukuzaki, H. (1983) Thromb. Res. 32, 183-188.
[27] Dawson, J., Thompson, N.T., Bonser, R.W., Hodson, H.F. and Garland, L.G. (1987) FEBS Lett. 214, 171-175.