differences in stimulus response coupling associated with $\alpha 1$ and $\alpha 2$ isoforms, as suggested by the greater sensitivity of the effect of low concentrations to ethylisopropylamiloride, an inhibitor of Na^+-H^+ exchange [5].

Acknowledgements—The authors thank Ms Anne Lebbe-Moureau for her skilful technical assistance. This work was supported by F.R.S.M. grant No. 3-9006-87 and by grant No. 89/95-135 from Actions de Recherche Concertées.

Laboratoire de Pharmacologie
Université Catholique de Louvain
B1200 Brussels
Belgium

FRANÇOIS NOËL*
MAURICE WIBO
THÉOPHILE GODFRAIND†

REFERENCES

1. Finet M, Godfraind T and Noël F, The inotropic effect of ouabain and its antagonism by dihydroouabain in rat isolated atria and ventricles in relation to specific binding sites. *Br J Pharmacol* **80**: 751–759, 1983.
2. Noël F and Godfraind T, Heterogeneity of ouabain specific binding sites and $(Na^+ + K^+)$-ATPase inhibition in microsomes from rat heart. *Biochem Pharmacol* **33**: 47–53, 1984.
3. Werdan K, Wagenknecht B, Zwissler B, Brown L, Krawietz W and Erdmann E, Cardiac glycoside receptors in cultured heart cells. II. Characterization of a high affinity and a low affinity binding site in heart muscle cells from neonatal rats. *Biochem Pharmacol* **33**: 1873–1886, 1984.
4. Grupp I, Im W-B, Lee CO, Lee S-W, Pecker MS and Schwartz A, Relation of sodium pump inhibition to positive inotropy at low concentrations of ouabain in rat heart muscle. *J Physiol (Lond)* **360**: 149–160, 1985.
5. Finet M and Godfraind T, Selective inhibition by ethylisopropylamiloride of the positive inotropic effect evoked by low concentrations of ouabain in rat isolated ventricles. *Br J Pharmacol* **89**: 533–538, 1986.
6. Akera T, Ng Y-C, Hadley R, Katano Y and Brody TM, High affinity and low affinity ouabain binding sites in the rat heart. *Eur J Pharmacol* **132**: 137–146, 1986.
7. Lelièvre LG, Charlemagne D, Mouas C and Swynghedauw B, Respective involvements of high- and low-affinity digitalis receptors in the inotropic response of isolated rat heart to ouabain. *Biochem Pharmacol* **35**: 3449–3455, 1986.
8. Sweadner KJ, Isozymes of the Na^+/K^+-ATPase. *Biochim Biophys Acta* **988**: 185–220, 1989.
9. Orlowski J and Lingrel JB, Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic α isoform and β subunit mRNAs. *J Biol Chem* **263**: 10436–10442, 1988.
10. Brandt N, Identification of two populations of cardiac microsomes with nitrendipine receptors: correlation of the distribution of dihydropyridine receptors with organelle specific markers. *Arch Biochem Biophys* **242**: 306–319, 1985.
11. Wibo M, Duong AT and Godfraind T, Subcellular location of semicarbazide-sensitive amine oxidase in rat aorta. *Eur J Biochem* **112**: 87–94, 1980.
12. Wibo M, De Roth L and Godfraind T, Pharmacologic relevance of dihydropyridine binding sites in membranes from rat aorta: kinetic and equilibrium studies. *Circ Res* **62**: 91–96, 1988.
13. McPherson GA, Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *J Pharmacol Methods* **14**: 213–228, 1985.
14. Sarmiento JG, Janis RA, Colvin RA, Triggle DJ and Katz AM, Binding of the calcium channel blocker nitrendipine to its receptor in purified sarcolemma from canine cardiac ventricle. *J Mol Cell Cardiol* **15**: 135–137, 1983.
15. Doyle DD, Kamp TJ, Palfrey HC, Miller RJ and Page E, Separation of cardiac plasmalemma into cell surface and T-tubular components. Distribution of saxitoxin- and nitrendipine-binding sites. *J Biol Chem* **261**: 6556–6563, 1986.
16. Brandt N and Bassett A, Separation of dihydropyridine binding sites from cardiac junctional sarcoplasmic reticulum. *Arch Biochem Biophys* **244**: 872–875, 1986.
17. Page E and Surdyk-Droske M, Distribution, surface density, and membrane area of diadic junctional contacts between plasma membrane and terminal cisterns in mammalian ventricle. *Circ Res* **45**: 260–267, 1979.

* On leave from Departamento de Farmacologia Basica e Clinica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

† To whom all correspondence should be addressed: Professor T. Godfraind, Laboratoire de Pharmacologie, Université Catholique de Louvain, UCL 73.50, Avenue E. Mounier, 73, B 1200 Brussels, Belgium.

Biochemical Pharmacology, Vol. 41, No. 2, pp. 315–318, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00
© 1990. Pergamon Press plc

Interference of xanthate compounds with phorbol ester TPA-induced changes of phospholipid metabolism: inhibition of prostaglandin production

(*Received* 6 *June* 1990; *accepted* 15 *September* 1990)

Certain xanthate compounds have been shown to act antivirally by inhibition of the transcription and replication of various DNA and RNA viruses without becoming incorporated into viral macromolecules and in the absence of interferon induction [1]. In addition, xanthate compounds have been shown to reverse growth kinetics and cell morphology of transformed cells to that of non-transformed phenotypes [2]. Moreover, these compounds very effectively

inhibit tumor promotion in mouse skin elicited by phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA*) [3]. These latter observations raised the possibility that xanthate compounds may interfere with signal transducing pathways, involving protein kinase C [4], particularly with TPA-effected changes in phospholipid metabolism [5].

Important cellular reactions to TPA consist in the activation of phospholipases C and A_2 which leads to the mobilization of biologically active phospholipid metabolites: diacylglycerol, respectively, arachidonic acid, the substrate for the production of prostaglandins and HETEs [6–8]. In order to learn more about the effectivity of xanthates we studied the interference of such compounds with responses to TPA elicited in HeLa cells. This model system mirrors several metabolic responses of mouse skin to treatment with phorbol esters [9, 10].

Materials and Methods

TPA was obtained from Sigma (Munich, F.R.G.) and was kept in acetone as a 5×10^{-4} M stock solution at −75°. The xanthate compounds D609 (MW 266) and D600 (MW 158) were synthesized by Merz & Co. (Frankfurt, F.R.G.) and kindly provided by Prof. G. Sauer (DKFZ, Heidelberg). Both compounds were kept in acetone as a 5×10^{-2} M stock solution. Arachidonic acid, prostaglandin E_2, 1,2- and 1,3-dioleoylglycerol were obtained from Sigma and BSA (bovine serum albumin, electrophoretically pure) from Biomol (Ilvesheim, F.R.G.). 5-, 12- and 15-HETE were obtained from Paesel (Frankfurt, F.R.G.). [1-^{14}C]Arachidonic acid (2.2 MBq/μmol), 1[1^{14}C]Palmitoyl-3-lysophosphatidylcholine (2.16 MBq/μmol) and *myo*-[2-^{3}H]inositol with PT6-271 (370 GBq/mmol) were obtained from Amersham Buchler (Braunschweig, F.R.G.).

Cloned HeLa cells were cultivated as monolayers as described previously [11]. For experiments 9×10^5 cells were transferred to plastic Petri dishes (Falcon 3.5 cm diameter); 18 hr later the cells were prelabeled with [1-^{14}C]arachidonic acid (7.4 kBq/mL) for 5 hr as described previously [10]. Then the medium was changed with BSA (0.5%)-containing MEM, in which the pH was adjusted to 6.9 to avoid inactivation of the xanthate compounds [1]. The cells were pretreated with the xanthates D609, D600 or acetone (0.2%; solvent control) for 30 min, and then TPA 10^{-7} M or acetone (0.2%) were added for the periods indicated. Analysis of the lipid metabolites released into the medium and the extraction of cellular lipids was performed as described previously [10]. Prostaglandins and HETE-products were identified with authentic standards. The cellular lipid extracts were separated on TLC-plates silicagel 60 (Sigma) in the solvent system benzol–chloroform–formic acid (80:15:5 v/v; [12]) with 1,2- and 1,3-dioleoylglycerol as standard. Radioactivity was determined by use of a Linear Analyzer (Berthold, Wildbad, F.R.G.). Because of 30% isomerization of 1,2-diacylglycerol to the 1,3-configuration during separation the counts of both labeled compounds were combined. Each experiment was performed twice. Values given are means of three dishes ± mean deviation.

Results

Treatment of HeLa cells with TPA resulted in the mobilization of diacylglycerol. This could be demonstrated with cells prelabeled for 5 hr with radioactive arachidonic acid. At 10^{-7} M TPA the increased mobilization became detectable after 20–30 min (Fig. 1A); it stayed elevated for 3–4 hr. Similar data were obtained after prelabeling of the cells with lysophosphatidylcholine (for 24 hr) thus indicating that a certain portion of diacylglycerol was derived from phosphatidylcholine (data not shown). In HeLa cells prelabeled with radioactive *myo*-inositol (for 24 hr) the treatment with TPA did not yield an increase in inositol phosphate (not shown) thus indicating that phosphatidylinositol was probably not the source of diacylglycerol.

Pretreatment of HeLa cells with 10^{-4} M D609 (27 μg/mL) for 30 min prior to addition of 10^{-7} M TPA did not influence the mobilization of diacylglycerol (Fig. 1A). A similar concentration (30 μg/mL) has been shown earlier to reduce TPA-effected mobilization of diacylglycerol in hamster embryo fibroblasts [5]. Cellular morphology (phase contrast microscopy) was not altered by xanthate during the time course of the experiments.

With respect to TPA-induced inflammation in tissues, a prerequisite for the promoting capacity of phorbol ester, the activation of phospholipase A_2 [8] resulting in the liberation of arachidonic acid plays an important role.

* Abbreviations: HETE, hydroxyeicosatetraenoic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; D600, sodium propyl-xanthate ($C_4H_7NaOS_2$); D609, potassium tricyclodican-9-yl-xanthate ($C_{11}H_{15}KOS_2$).

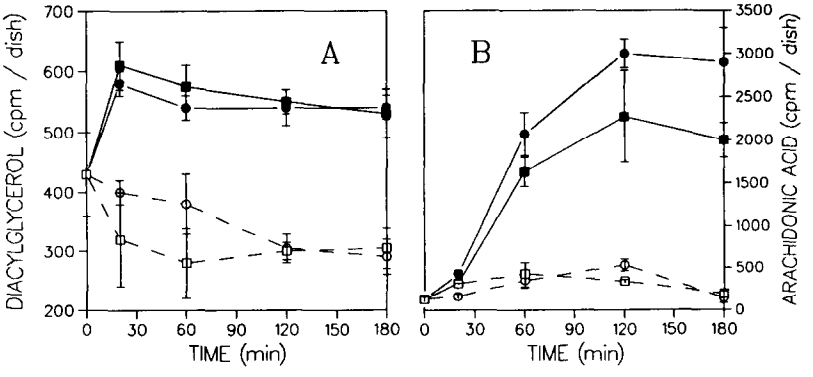

Fig. 1. Effect of the xanthate D609 on the TPA-induced diacylglycerol formation (A) and arachidonic acid release (B). HeLa cells were cultivated for 18 hr and after prelabeling with [1-^{14}C]arachidonic acid for 5 hr the medium was changed to BSA-(0.5%)-containing MEM (pH 6.9). The cells were pretreated with 10^{-4} M D609 (27 μg/mL; ○, ●) or acetone as solvent (0.2%; □, ■) for 30 min and then TPA 10^{-7} M (●, ■) or acetone (0.2%; ○, □) was added for the periods indicated. Diacylglycerol formation (A) in the cells and arachidonic acid release into the medium (B) was analysed as described in Materials and Methods.

Arachidonic acid is the substrate for the formation of a number of biologically active compounds including prostaglandins. Using 10^{-4} M D609 it was not possible to influence TPA-effected liberation of arachidonic acid (Fig. 1B). In a number of experiments even an increase in arachidonic acid accumulation was observed after 3 hr.

The predominant prostaglandin released by HeLa cells after interaction with TPA was prostaglandin E_2 which becomes measurable at 30 min and reaches a maximum at 2 hr [10]. Under the conditions used D609 inhibits the production of prostaglandin E_2 as shown in Fig. 2A. After a small initial increase at 2 hr prostaglandin E_2 production became totally blocked. In such experiments in which a TPA-effected increase in HETE production was measurable, D609 inhibited the HETE production as well.

The half maximal efficiency of D609 has been seen at approximately 3×10^{-5} M after a 3 hr incubation with TPA (Fig. 3A). In contrast to D609 another xanthate called D600 (propylxanthate) has been demonstrated not to interfere with viral infections nor to reverse the transformed phenotype of cells *in vitro* [13]. With respect to the biochemical results obtained in this study, however, D600 had a comparable activity. It interfered with the TPA-induced prostaglandin production like D609. The half maximal inhibitory activity of D600 on TPA-effected prostaglandin production was also observed at approx. 3×10^{-5} M (Fig. 3A). At high concentrations (10^{-4} M) neither D609 nor D600 influenced TPA-effected arachidonic acid liberation to a significant extent (Fig. 3B).

Taken together, these data demonstrate that both compounds of the xanthate family did not influence the TPA-effected release of arachidonic acid but inhibited greatly the utilization of the substrate for the production of prostaglandins and HETEs.

Discussion

Xanthates such as Tricyclodecan-9-yl xanthate (D609) have been found to be potent inhibitors of TPA-induced tumor promotion in mouse skin, an effect which strictly depends on the bulky lipophilic cycloalkyl residue, since the short chain alkyl derivative propylxanthogenate (D600) was completely inactive [3]. The mechanism of D609's antipromoting activity is unknown. As a highly lipophilic agent D609 could exert its inhibitory activity by interfering with TPA-induced alterations of cellular lipid metabolism including the phosphatidylcholine cycle [7] and the arachidonic acid cascade [8]. In fact, D609 has been shown to inhibit TPA-induced activation of a phosphatidylcholine-

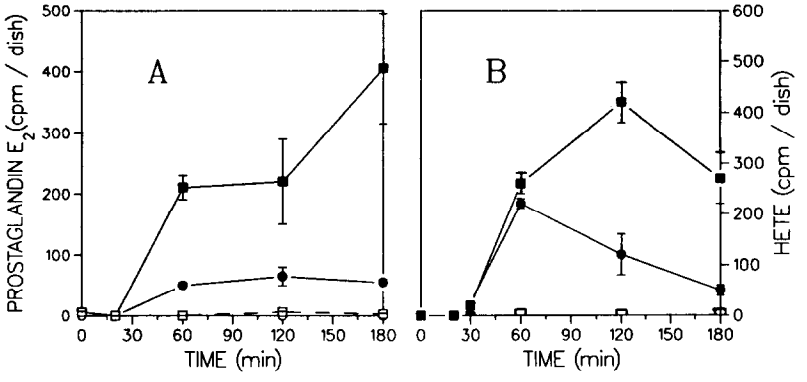

Fig. 2. Influence of D609 on the TPA-induced prostaglandin E_2- (A) and HETE-production (B). HeLa cells were incubated with 10^{-4} M D609 (27 μg/mL; ○, ●) or acetone (0.2%; □, ■) and treated with TPA 10^{-7} M (●, ■) or acetone (0.2%; ○, □) under the conditions described in Fig. 1. The prostaglandin E_2- (A) and total HETE-production (B) was analysed as described in Materials and Methods.

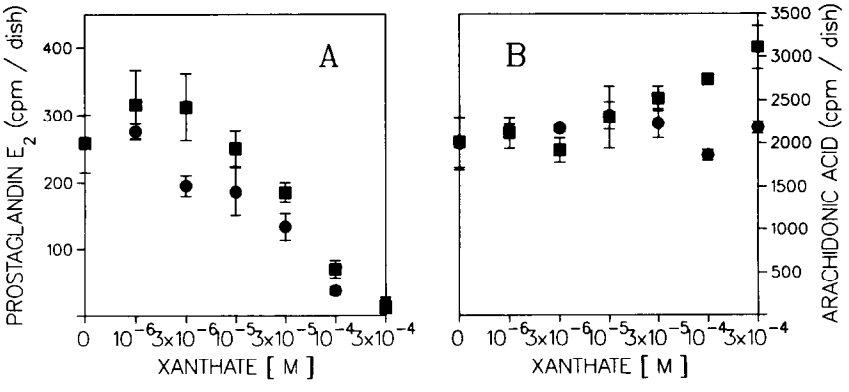

Fig. 3. Influence of the xanthates D609 and D600 on the TPA-induced prostaglandin E_2-synthesis (A) and the arachidonic acid release (B) in the medium of HeLa cell cultures. HeLa cells were prelabeled with [1-^{14}C]arachidonic acid for 5 hr and after medium change with BSA-(0.5%)-containing MEM (pH 6.9) the cells were pretreated with varying doses of D609 (■) or D600 (●) for 30 min. Then TPA 10^{-7} M was added to the cultures for 3 hr. Prostaglandin E_2- (A) and arachidonic acid (B) were analysed in the medium as described in Materials and Methods.

specific phospholipase C activity in resting A431 cells and hamster embryo fibroblasts [5]. Using HeLa cells as an exclusively replicating model system, an inhibitory effect of D609 on TPA-induced diacylglycerol release from phosphatidylcholine could not be observed. In addition, TPA-induced deacylation of phospholipids and concomitant liberation of arachidonic acid—the rate limiting step of eicosanoid biosynthesis—was found to be slightly increased rather than inhibited. This is most probably due to D609-effected inhibition of arachidonic acid metabolism via both the cyclooxygenase and the lipoxygenase pathways; an inhibition of acyl transferase activity could be also involved. Interestingly, both D609 and its less lipophilic analogue D600, turned out to be active, exhibiting half maximal inhibition of PGE_2 synthesis at a concentration of 3×10^{-5} M. This may indicate that the dithiocarbonic acid part of the molecule is responsible for the inhibitory activity.

In comparison with other inhibitors of prostaglandin production, xanthates turn out to be equipotent with non-acidic non-steroidal anti-inflammatory drugs such as paracetamol (IC_{50} in macrophages 7.9×10^{-5} M; Ref. 14) but less active than the potent acidic non-steroidal anti-inflammatory drugs such as indomethacin in macrophages (IC_{50} 3×10^{-8} M; Ref. 15). If this hitherto unknown activity of xanthates is involved in the antipromoting and the antiviral action of some of them remains an open question.

Acknowledgements—Work supported by the Deutsche Forschungsgemeinschaft.

M. KASZKIN*
G. FÜRSTENBERGER†
V. KINZEL*‡

* *Institute of Experimental Pathology and*
† *Institute of Biochemistry*
German Cancer Research Center
Im Neuenheimer Feld 280
D-6900 Heidelberg
Federal Republic of Germany

REFERENCES

1. Sauer G, Amtmann E, Melber K, Knapp A, Müller K, Hummel K and Scherm A, DNA and RNA virus species inhibited by xanthates, a class of antiviral compounds with unique properties. *Proc Natl Acad Sci USA* **81**: 3263–3267, 1984.
2. Amtmann E, Müller K, Knapp A and Sauer G, Reversion of bovine papilloma virus-induced transformation and immortalization by a xanthate compound. *Exp Cell Res* **161**: 541–550, 1985.
3. Fürstenberger G, Amtmann E, Marks F and Sauer G, Tumor prevention by a xanthate compound in experimental mouse skin tumorigenesis. *Int J Cancer* **43**: 508–512, 1989.
4. Müller-Decker K, Doppler C, Amtmann E and Sauer G, Interruption of growth signal transduction by an antiviral and antitumoral xanthate compound. *Exp Cell Res* **177**: 295–302, 1988.
5. Müller-Decker K, Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase C-type reaction. *Biochem Biophys Res Commun* **162**: 198–205, 1989.
6. Besterman JM, Duronio V and Cuatrecasas P, Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for generation of a second messenger. *Proc Natl Acad Sci USA* **83**: 6785–6789, 1986.
7. Daniel LW, Waite M and Wykle RL, A novel mechanism of diglyceride formation: 12-*O*-tetradecanoylphorbol-13-acetate stimulates the cyclic breakdown and resynthesis of phosphatidylcholine. *J Biol Chem* **261**: 9128–9132, 1986.
8. Levine L, Arachidonic acid transformation and tumor production. *Adv Cancer Res* **35**: 49–79, 1981.
9. Kinzel V, Kreibich G, Hecker E and Süss R, Stimulation of choline incorporation in cell cultures by phorbol derivatives and its correlation with their irritant and tumor-promoting capacity. *Cancer Res* **39**: 2743–2750, 1979.
10. Espe U, Fürstenberger G, Marks F, Kaszkin M and Kinzel V, Early changes in the arachidonic acid metabolism of HeLa cells in response to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and related compounds. *J Cancer Res Clin Oncol* **113**: 137–144, 1987.
11. Kinzel V, Richards J and Stöhr M, Early effects of the tumor promoting phorbolester 12-*O*-tetradecanoyl-phorbol-13-acetate on the cell cycle traverse of asynchronous HeLa cells. *Cancer Res* **41**: 300–305, 1981.
12. Takuwa N, Takuwa Y and Rasmussen H, A tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate, increases cellular 1,2-diacylglycerol content through a mechanism other than phosphoinositide hydrolysis in Swiss-mouse 3T3 fibroblasts. *Biochem J* **243**: 647–653, 1987.
13. Sauer G, Amtmann E and Schalasta G, Xanthogenate, eine Verbindungsklasse mit gezielter Wirkung auf transformierte Zellen. In: *Die Zellmembran als Angriffspunkt der Tumortherapie; Aktuelle Onkologie* (Eds. Unger C, Eibl H and Nagl GA), Vol. 34, pp. 124–135. W. Zuckschwerdt, München, 1987.
14. Brune K, Rainford KD, Wagner K and Peskar BA, Inhibition by anti-inflammatory drugs of prostaglandin production in cultured macrophages. *Naunyn Schmiedebergs Arch Pharmacol* **315**: 269–276, 1981.
15. Pentland AP and Needleman P, Modulation of keratinocyte proliferation *in vitro* by endogenous prostaglandin synthesis. *J Clin Invest* **77**: 246–251, 1986.

‡ To whom correspondence should be addressed.