Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 532-539

DIACYLGLYCEROLS RELEASE LH: STRUCTURE-ACTIVITY RELATIONS REVEAL A ROLE FOR PROTEIN KINASE C

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Received November 30, 1984

Summary: A series of diacylglycerols were synthesized with varying lengths and substituents in order to establish the structure-activity relationship between each with activation of protein kinase C and stimulation of a biological response system (pituitary luteinizing hormone release). This approach enables distinction between actions mediated by direct activation of protein kinase C and those due to other, presumably nonspecific, actions. The ability of diacylglycerols to function as regulators of a biological response system (pituitary luteinizing hormone release) and of protein kinase C was investigated with a series of sn-1,2 diacylglycerols containing fatty acids 4-10 carbons in length and with analogs in which the 3' hydroxyl was replaced with a chloro, hydrogen, or sulfhydryl moiety. Several diacylglycerols stimulated LH release in a saturable, time and dose dependent manner that was independent of extracellular calcium. Dioctanoylglycerol (diC_8) was the most effective of the diacylglycerols tested; 3' analogs lacking the hydroxyl were inactive. The diacylglycerols activated protein kinase C *in vitro* whereas the 3' analogs did not. These data implicate protein kinase C in the mechanism of LH release, demonstrate that unsaturated fatty acyl moieties within the diacylglycerol are not required for protein kinase C activation, and establish diacylglycerol-protein kinase C structure-function relationships that should prove useful for investigations in other systems.

Investigation of the mechanism by which gonadotropin releasing hormone (GnRH) occupancy of its receptor stimulates pituitary luteinizing hormone (LH) release has largely focused on the mobilization of extracellular Ca^{2+} and on the intracellular events for which Ca^{2+} fulfills the requirements of a second messenger (1). Phorbol esters, which bind and activate protein kinase C, also stimulate LH secretion (2,3) by a mechanism independent of extracellular Ca^{2+}.

Abbreviations used are: GnRH, gonadotropin releasing hormone; diC_4, *sn* 1,2-dibutyryl-glycerol; diC_6, *sn* 1,2-dihexanoylglycerol; diC_8, *sn*-1,2-dioctanoylglycerol; diC_{10}, *sn* 1,2-didecanoyl glycerol; $diC_{18:1}$, *sn* 1,2-dioleoylglycerol; OAG, *sn* 1-oleoyl-2-acetylglycerol; LPC, 1-oleoylglycerolphosphorylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; LH, luteinizing hormone; 199/BSA, Medium 199 containing 0.3% bovine serum albumin; BSA, bovine serum albumin

0006-291X/85 $1.50

Diacylglycerol has been implicated as the endogenous regulator of protein kinase C since it binds to the same site as the phorbol esters (4,5), activates the enzyme (4,6), and appears to arise from turnover of polyphosphoinositides in response to stimulation (7). Although the structural requirements for protein kinase C activation were not reported, Nishizuka and co-workers concluded on the basis of available *in vitro* studies that an unsaturated fatty acyl moiety within the diacylglycerol was required (8). Diacylglycerols containing long chain fatty acids were not active biologically when added to cells, however 1-oleoyl-2-acetylglycerol (OAG) activated protein kinase C *in vitro* (9) and caused secretion from platelets (9) as well as secretion and O_2^- production by neutrophils (10). When these studies were begun, OAG was the only diacylglycerol analog known to elicit biological responses when added to cells.

The objectives of the present study were to assess whether diacylglycerols would function as regulators of LH release, to establish whether an unsaturated fatty acyl moiety within the diacylglycerol is required for protein kinase C activation, and to probe the diacylglycerol-protein kinase C and diacylglycerol LH release structure function relationships with diacylglycerols and their analogs. These studies were undertaken to directly test the hypothesis that diacylglycerols function as intracellular regulators.

METHODS: *Pituitary cell cultures* were prepared from pituitaries of female weanlings (Harlan Sprague-Dawley, Indianapolis, IN) in Medium 199 containing 0.3% BSA. After two days in culture, the cells were washed and the medium was replaced with fresh 199/BSA containing lipid at the indicated concentration. After 3 hours the medium was removed and LH determined by RIA (11). Values are expressed in terms of NIH standard RP1 and adjusted for the numbers of cells by DNA determination (12). *Lipids* were synthesized as follows: *sn 1,2, diacylglycerols*: PC (100 µmol) of the appropriate acyl chain length (Avanti Polar Lipids) was digested with phospholipase C (Sigma, *Bacillus cereus*, 50 units) for 2 hours in a two phase ether/water system described (13). The ether was evaporated under a stream of nitrogen and lipids were extracted with chloroform-methanol (14), redissolved in chloroform and loaded on a small column (1 ml bed) of silica gel in chloroform. The column was washed with 2 ml chloroform then twice with chloroform:methanol, 4:1. The eluates were pooled and dried under nitrogen, and the purified diacylglycerols were dissolved in chloroform and stored at -20° C. *sn-1-oleoyl-2-acetylglycerol*: Dioleoylphosphatidylcholine was digested with phospholipase A_2 from *Crotalus adamanteus* venom, (Miami Serpentarium Laboratories) essentially as described by Kates (15). The oleic acid and sn-1-oleoylglycerophosphorylcholine were separated on silica gel. The lysophosphatidylcholine was dissolved in 2 ml chloroform to which 0.25 ml each of acetic anhydride and pyridine were added. This mixture was incubated at room temperature in the dark overnight. Methanol (1 ml) was added to consume

excess acetic anhydride. After 3 hours, solvents were largely removed under nitrogen, and excess acetic acid and pyridine removed by chloroform-methanol extraction and washing of the chloroform phase successively with neutral and acidic upper phase. sn-1-oleoyl-2-acetylglycerol was prepared by digesting the sn-1-oleoyl-2-acetylglycerophosphorylcholine with phospholipase C as described above. Deoxy-, chloro-, and sulfhydryl- analogs of diC8: To 1 ml heptane were added 0.25 ml pyridine, 600 µmol octanoic anhydride (16), and 150 µmol of either 1,2 propanediol, 3-chloro-1,2-propandiol (both from Sigma), or 1-thiomethyl-1-thioglycerol (17). After 20 hours at room temperature the solvents were evaporated and pyridine removed by acidic chloroform-methanol extraction. The lipids were dissolved in heptane and fatty acid was extracted with 50 mM NaOH in 50% ethanol. The resulting heptane phase containing the diacyl derivative and traces of monoacyl and other products, was chromatographed on 0.8 x 5 cm column of silica gel in heptane then eluted with chloroform:heptane, 2:3. The first material to elute was the pure diacyl derivative, which was dissolved in chloroform and stored at -20°C. The thiomethyl protecting group was removed from the sulfhydryl analog by reduction with a four-fold excess of dithiothreitol in methanol at 75° C for 24 hours. The methanol was evaporated, and the lipid was separated from dithiothreitol by partitioning between water and heptane. The heptane phase, containing the lipid, was chromatographed as described above. The criterion for purity of diacylglycerol and its analogs was migration as a single spot on Silica Gel 60 thin layer chromatography plates (Merck) developed in heptane:diethyl ether:acetic acid (25:75:1, v:v:v) and detected by spraying with 33% sulfuric acid and charring at 180° C. Protein kinase C was measured in the presence of the indicated concentration of each of the indicated lipids as previously described (4). Briefly, 2 µg of purified extract from rat brain was incubated in 250 µl 20 mM Tris-HCl, pH 7.5 containing 2 µM Ca^{2+}, 10 µM ^{32}P-ATP (gamma)(420,000 cpm), 10 mM magnesium acetate, 200 µg histone IIIS (Sigma), 20 µg/ml PS for 10 min at 24° C. The reaction was stopped by addition of 1 ml 25% trichloroacetic acid and 1 ml (500 ug) BSA, then filtered through a Millipore filter (type HA) which was washed with 5 ml of 25% TCA, dried, and counted in 10 ml of a water accepting scintillation fluid. Ca^{2+} independent kinase activity was measured in the presence of 1 mM EGTA and was less than 10% of the Ca^{2+}-dependent activity.

RESULTS AND DISCUSSION: We selected for study a number of the diacylglycerols containing short chain saturated fatty acids since those with long chains present problems of solubility and delivery to living cells. Several of the former group stimulated LH release in a saturable, time and dose dependent manner (Figure 1). For all these diacylglycerols, extracellular Ca^{2+} was not required for release of LH. sn-1,2-dioctanoylglycerol (diC_8) was the most effective of the diacylglycerols tested. sn 1,2-dihexylglycerol (diC_6) was less efficacious and had a lower potency than diC_8 (Figure 1). DiC_6 and diC_8 were more potent than OAG, which has been used (18) to activate protein kinase C in cells (7). sn 1,2-didecanoyl glycerol (diC_{10}) consistently stimulated the release of small amounts of LH whereas sn 1,2-dibutyrylglycerol (diC_4) and sn 1,2-dioleoylglycerol ($diC_{18:1}$) were essentially inactive over the concentration range tested. Clearly a number of diacylglycerols containing saturated short chain

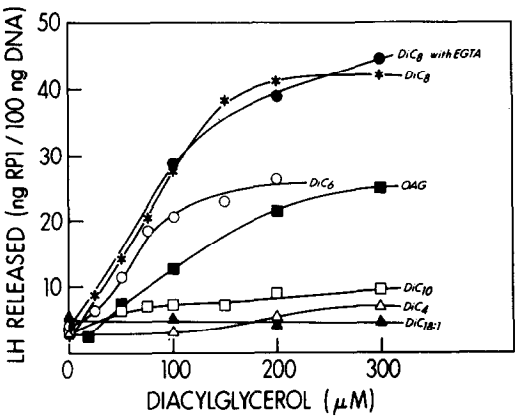

Fig. 1: Effect of diacylglycerol on LH release from pituitary cultures. Cultures from pituitaries of female weanlings were incubated with the indicated lipid as described in Methods and LH released during 3 hours determined by RIA.

fatty acids were active. Recently, diC_{10} was reported to promote phosphorylation of a 40,000 dalton protein in human platelets (19). The decreased activity with diC_{10} and the total absence of activity with $diC_{18:1}$ probably reflects physical properties which prevent effective delivery.

The amount of LH released by a saturating dose of diC_8 (200 μM) was 77% of the amount released in response to a maximally stimulating dose of GnRH (1 μM). During a 3h incubation period 40-50% of the total cellular LH (assessed following detergent solubilization) was released by GnRH (20). The remaining LH was not released upon further incubation (20). This characteristic time course of LH release in response to GnRH was also observed in response to diC_8. The characteristic time course for LH release, the continued ability of cells to exclude trypan blue, retain normal morphology as viewed under phase and Nomarski optics, and lack of release of malate dehydrogenase activity, allowed us to exclude cell lysis in response to the diacylglycerols.

Since the pituitary cells are cultured in medium containing 3 mg/ml BSA and since fatty alcohols are known to bind to BSA, we investigated the possibility that albumin would modulate the release of LH in response to diC_8. Neither the absence of albumin nor its 10-fold elevation prevented diC_8 dependent LH release. In contrast, when lysophosphatidylcholine, an amphipath with well-

known detergent properties, was tested for its ability to promote LH release, 100-200 uM caused total release of LH at 0 and 1 mg/ml BSA. However, at 30 mg/ml BSA, LH release was totally suppressed. Thus the stimulation of LH release by diC_8 is distinct from that caused by detergent action.

Further structure-function analysis of diacylglycerol dependent release of LH focused on the 3' hydroxyl group. We prepared diC_8 analogs in which the hydroxyl group was replaced by hydrogen, chloro, of sulfhydryl moieties. None of these analogs stimulated LH release alone or antagonized release in response to diC_8 up to 300 μM. Accordingly, the hydroxyl group is essential in promoting LH release. Other related compounds tested at concentrations up to 300 uM that were ineffective in releasing LH were: sodium dodecyl sulfate, n-decanol, Zwittergent 3-10, octylglucoside, and oleic, palmitic, and arachidonic acids.

We next examined the ability of these diacylglycerols and analogs to activate protein kinase C *in vitro*. Owing to the sensitivity of the assay, the pooled pituitaries of over 1,000 rats produced only enough protein to clearly demonstrate the presence of protein kinase C. Since larger amounts of tissue were required to determine the diacylglycerol structure-function relationships, protein kinase C purified (4) from rat brain was used. At 2 μM Ca^{2+} and 20 μg/ml phosphatidylserine (PS), diC_8 activated protein kinase C (Figure 2). OAG and diC_{10} were similar to diC_8 whereas diC_6 was about 10-fold less potent. DiC_4 caused activation of protein kinase C at concentrations exceeding 100 uM where the molar ratio of diC_4 to PS was greater than 3:1. Clearly, diacylglycerols containing saturated fatty acids acids activate protein kinase C. The earlier conclusion (8) that an unsaturated fatty acyl moiety with the diacylglycerol was required for activation may have reflected the influence of unsaturation on the physical properties of the lipid. The chloro, sulfhydryl, and deoxy analogs of diC_8 were inactive.

$DiC_{18:1}$ was indistinguishable from diC_8 and OAG in its ability to activate protein kinase C *in vitro* (Figure 2), when it was sonically dispersed with PS.

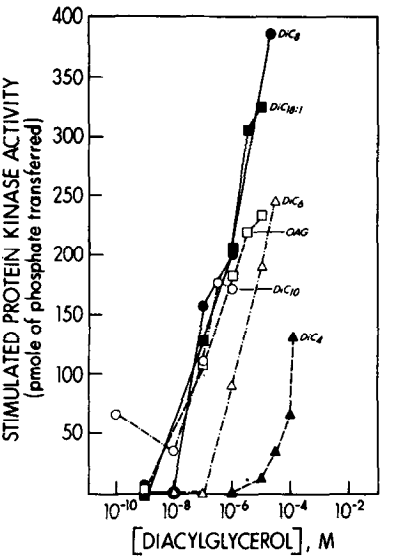

Fig. 2: Lipid stimulation of protein kinase C *in vitro*. Protein kinase was measured in the presence of the indicated concentration of each of the indicated lipids as described in Methods.

However $diC_{18:1}$ was totally ineffective in promoting LH release (Figure 1). The physical delivery of diacylglycerols to the cells for protein kinase C activation and, thereby, promotion of LH release, underlies the dependence on fatty acid chain length observed (Figure 1). The fatty acid chain length must be short enough to permit sufficient solubility for delivery, yet long enough to partition into the bilayer to cause activation of protein kinase C. The partitioning requirement would account for the failure of diC_4 to function as well as diC_6 and diC_8. At the level of diC_{10} solubility would be decreasing rapidly so that delivery becomes a problem; with $diC_{18:1}$, the delivery problem is so severe that no activity is observed. *In vitro* diC_8, diC_{10}, and $diC_{18:1}$ activated protein kinase C identically when the diacylglycerol were delivered by cosonication with PS. Hence diC_8 appears to possess physical properties consistent with efficient cellular delivery and effective activation of protein kinase C.

The polyphosphoinositides appear to play a role in a number of diverse biological systems since stimulation of these systems, including GnRH-stimulated

LH release, results in metabolism of polyphosphoinositides and the formation of inositol triphosphates and diacylglycerols (for review, 7). The observations in the present work that diacylglycerols containing short chain saturated fatty acids can both stimulate LH release from pituitary cells and activate protein kinase C whereas the analogs lacking the 3' hydroxyl group were inactive argues that protein kinase C plays a role in regulation of LH release. Because it appears that extracellular Ca^{2+} is mobilized in response to GnRH receptor occupancy by an agonist (21), diacylglycerols may exert their action by lowering the K_m of protein kinase C for Ca^{2+} as has been reported to occur in vitro (8,22).

Diacylglycerols containing short chain fatty acids such as diC_8 should prove useful for other studies involving protein kinase C, since they are easy to prepare, activate the enzyme in vitro, and can be effectively delivered to intact cells. In consideration of the fact that phorbol esters are tumor promoters and diacylglycerols containing short chain fatty acid mimic the effects of phorbol-esters on pituitary cells and on protein kinase C, these diacylglycerols should be handled with precautions pending complete understanding of their biological actions.

ACKNOWLEDGMENTS:

This work was partially supported by NIH HD13220 and The Mellon Foundation (PMC), NIH CA35680 and The Chicago Community Trust/Searle Scholars Program (JEN), and NIH AM20205 (RMB).

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