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BIPHASIC GENERATION OF DIACYLGLYCEROL BY ANGIOTENSIN AND PHORBOL ESTER IN BOVINE ADRENAL CHROMAFFIN CELLS

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Summary. Stimulation of angiotensin receptors in bovine adrenal medullary cells with Sar^1-angiotensin II increased diacylglycerol levels in a biphasic fashion. An initial peak occured at 3 min and an increase was observed again at 60 min and even at 18 hrs. Phorbol 12-myristate 13-acetate produced a similar pattern of increase in diacylglycerol levels. Both the angiotensin analog and the phorbol ester also increased the release of (^{3}H)choline into the culture medium from prelabelled cells. The long-term diacylglycerol production could be derived from phosphatidylcholine rather than from the phosphoinositides. The latter may be the source of the angiotensin stimulated initial production of diacylglycerol and activation of PKC. Activated PKC then turns on the continuous production of DAG which maintains PKC in an active state for long periods of time in the presence of the peptide.

Long-term incubation of BAM cells with AII persistently activates PKC (1). Under these conditions mRNA levels encoding the catecholamine synthetizing enzymes, tyrosine hydroxylase TH and phenylethanolamine-N-methyltranseferase (2) are increased. Sphingosine, a rather specific inhibitor of PKC (3,4), inhibits the increases in the mRNA levels, suggesting the involvement of PKC in mediating the effect of AII (2). Activation of PKC in BAM cells may occur through several different mechanisms; AII activates the PLC-dependent inositol phosphate cascade (5), which produces two intracellular second messengers, DAG and IP3 (6). DAG activates PKC (7), and causes it to translocate from soluble to particulate fractions (8). IP3 releases Ca^{2+} from intracellular stores (9). Also high levels of free intracellular Ca^{2+} in BAM cells can translocate PKC from the cytosol to the cell membranes (10). However, high intracellular Ca^{2+}-levels are transiently generated by AII, and upon persistent incubation with AII, the Ca^{2+} levels return to nearly resting levels (11). DAG is known to reduce the Ca^{2+} requirement of PKC activation to basal levels of free intracellular Ca^{2+} (12). On the

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ABBREVIATIONS: BAM, bovine adrenal medullary; DAG, diacylglycerol; IP3, inositoltrisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphoinositide(s); PIP2, phosphatidylinositolbisphosphate; PKC, protein kinase C; PI, phosphatidylinositol; PL, phospholipid; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; S^1-AII, sar_1-angiotensin II.

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other hand, similar to the generation of IP3 (11), also production of DAG from PIP2 also may be transient in BAM cells. In recent years, data has accumulated suggesting that receptor activated hydrolysis of PC also is a source of DAG (13). Therefore, we have measured the effects of AII and PMA on DAG levels in BAM cells, with special emphasis on the long-term effects.

Materials and Methods

PMA, S^1-AII, calf thymus histone, EGTA, EDTA, DAG, phosphatidylserine, DMEM/F12 medium, and Percoll were from Sigma Chemical Co. (St. Louis Mo). Fetal calf serum was from Gibco BRL Res. Products (Grand Island, NY). Collagenase type B was purchased from Boehringer Mannheim Biochemicals, (Indianapolis, IN), (^{3}H)choline and (^{32}P)-ATP from New England Nuclear/ DuPont, (Boston, MA), and the DAG-kinase kits from Lipidex (Pelham, New York).

Cell culture: Primary cultures of BAM chromaffin cells were prepared by the method of Wilson and Viveros (14) with minor modifications (1). Bovine adrenal medullae were digested with collagenase type B and the dispersed BAM cells were then isolated by centrifugation through a percoll gradient. After subsequent washings the cells were mixed in DMEM/F12 medium containing 10% fetal calf serum and antibiotics. The cells were plated at a density of 0.5-0.6x10^6 cells/cm^2 on culture plates and were kept in an incubator in humidified air/5% CO_2-atmosphere at 37°C for 3-5 days thereafter. Two days after plating the medium was changed to serum free medium. Detailed information regarding the purity of the BAM cells was described in a previous report (1).

Preparation of drug solutions: S^1-AII was dissolved in 10mM acetic acid/15% ethanol/water (w/w) and PMA was dissolved in dimethyl sulfoxide. Prior to use, the stock solutions were diluted with serum free cell culture medium (37°C) to obtain the final concentrations. In each case, the corresponding vehicle was used for control treatments.

Diacylglycerol assay: DAG concentrations were determined by the method of Preiss *et al.* (15) as outlined below. Lipids were extracted using the method of Bligh and Dyer (16) and total phosphate in each sample was measured by the method of Lazarus and Chou (17). For the determination of DAG, the dried lipid sample was solubilized in 20 µl of 7.5 % octyl-ß-D-glucoside/25 mM sn-1,2-dioleoyl-3-phosphatidyl-glycerol solution. The final reaction mixture contained 72 mM imidazole HCl, pH 6.6, 50 mM LiCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 50 µg/ml E.Coli DAG-kinase and 1 mM mixture of cold ATP plus (^{32}P)ATP. The (^{32}P)ATP was added to each tube to start the reaction; after 30 min at room temperature the reaction was terminated by the addition of 3 ml chloroform:methanol (1:2, v/v). Following lipid extraction, the dried sample of the organic phase was redissolved in 50 µl of chloroform:methanol (95:5, v/v) and 20 µl of the sample was spotted on the TLC plate. The plates were developed with chloroform/acetone/methanol/acetic/acid/water (10:4:3:2:1, v/v/v/v/v), for 90 min. After autoradiography, the areas corresponding to radioactivity in the PA band were scraped and counted in 4 ml Aquasol. The results are reported as the percent change in the PA/PL-ratio.

(^{3}H)Choline release: BAM cells were incubated for 24-48 hours with (^{3}H)choline (200,000 cpm/0.5x10^6cells/0.5ml) in serum-free DMEM/F12 medium. Unincorporated (^{3}H)choline was removed by washing the cells six to eight times. Aliquots of media were taken for radioactivity measurements at the indicated times and normalized to total (^{3}H)choline at the end of the experiment. Agonist stimulations relative to basal release are presented.

Statistics: Means, standard deviations and standard errors of the mean were calculated. One-way analysis of variance was used to test the overall statistical significance. Fisher's least significant difference test was used when three or more groups were compared. $P < 0.05$ was considered statistically significant.

Results

The peptide S^1-AII (0.2 µM) stimulated a biphasic DAG production. DAG-levels undervent significant increases of 43.1% and 58.7% after 3 and 60 min of stimulation, respectively. No significant

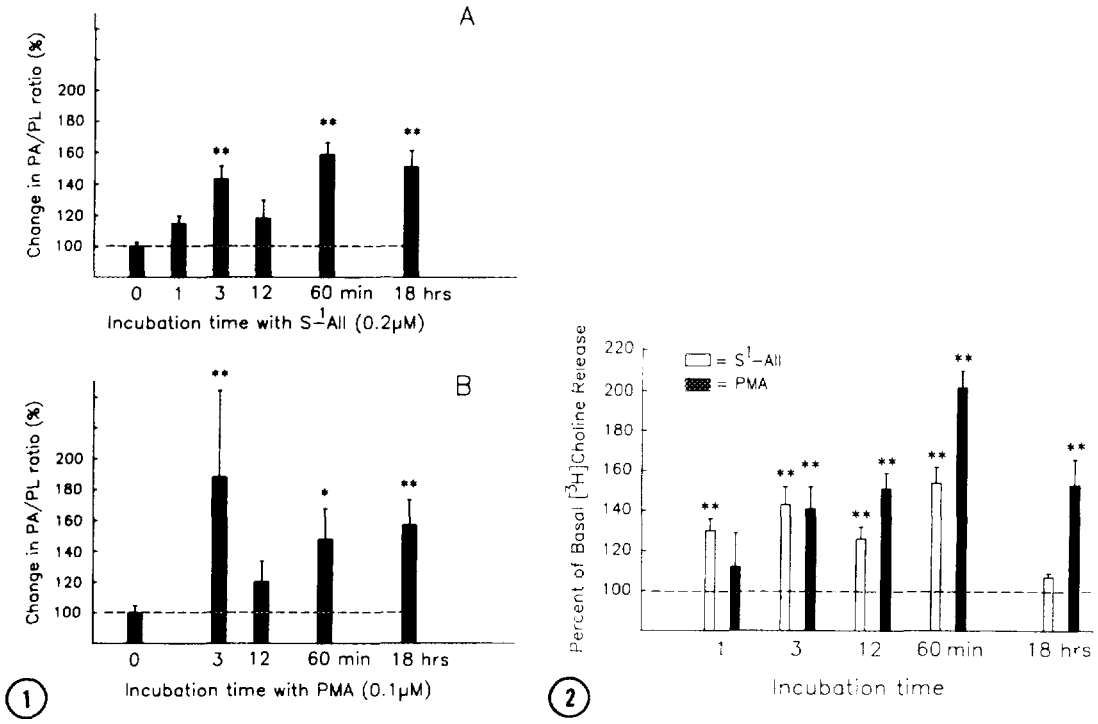

Figure 1. **A.** Time-course of the effect of S^1-All (0.2 μM) on diacylglycerol (DAG) levels in BAM cells. n = 7-39 (number of wells in each group) from five different cell preparations. **B.** Time-course of the effect of PMA (0.1 μM) on diacylglycerol (DAG) levels in BAM cells. n=5-23 (wells/group) from five different cell preparations. 5 x 10^5 cells/sample were used. The results are expressed as changes in PA/total phospholipid (PL) ratio, control = 100%. Mean ± SEM. Statistics: * p< 0.05 and ** p< 0.01 vs corresponding control.

Figure 2. Time-course of the effects of S^1-All (0.2 μM) and PMA (0.1 μM) on (^{3}H)choline release from (^{3}H)choline-labelled BAM cells. The results are expressed as percent of basal (=100%) (^{3}H)choline in the cell culture medium. Mean ± SEM, n = 7-9 (wells/group), data from 3 different cell preparations. Statistics: ** P< 0.01 vs corresponding control.

increases in DAG were observed after 1 and 12 min of incubation (Fig.1A). Furthermore, continuous incubation with S^1-All (0.2 μM) increased DAG levels 51.3 % even after 18 hrs of stimulation (Fig.1A).

The phorbol ester, PMA (0.1 μM), mimicked the effects of S^1-All on DAG levels (Fig.1B). It increased the DAG levels by 87.9%, 47.7% and 57% after 3 min, 60 min and 18 hrs of continuous incubation, respectively (Fig. 1B). As was observed with S^1-All, DAG-levels after 12 min stimulation with PMA were not increased. In (^{3}H)choline labelled cells, both S^1-All (0.2μM) and PMA (0.1 μM) increased the release of (^{3}H)choline at 1 hr (Fig. 2).

Discussion

This work demonstrates that All induces a sustained elevation of DAG levels in BAM cells. Thus, the long-term activation of PKC by All (1) is closely associated with and probably caused by persistent DAG formation. S^1-All increases DAG levels in a biphasic manner. The initial peak occurs at

3 minutes while the sustained elevation appears at 1 hr, and appears to last for at least 18 hrs. This is in accord with the biphasic time course of All-induced DAG formation in vascular smooth muscle cells which was associated with an early monophasic rise in IP3 and later formation of IP2 and IP (19). The activation of PLC in BAM cells by All leads to the formation of inositol phosphates (5,11). When PIP2 is hydrolyzed to IP3, DAG is formed as well (6). All-induced formation of IP3 in BAM cells peaks within seconds (11), but the DAG levels are significantly increased only at 3 min. A similar relationship between IP3 and DAG formation exists in PC12 pheochromocytoma cells when stimulated by carbachol or bradykinin (20). However, Plevin and Boarder (5) observed a linear accumulation of inositol phosphates in BAM cells during continual incubation with All over a period of 80 min. Therefore, it is possible that the initial peak in DAG-levels is derived from PLC-dependent breakdown of PIP2 where as the later increase in DAG-levels is derived from other precursors, such as PIP and PI.

Several studies suggest that PKC can play a role in regulating DAG production. PKC activation causes desensitation of agonist-induced PLC activation in BAM cells (21, 22). Therefore, it can be hypothesized that the All-induced PKC activation inhibits the effect of the peptide on PI-specific PLC as well. This would inhibit DAG-production and cause DAG levels to return to baseline after an initial burst of production. If this holds true, then the later sustained increases DAG may be derived from other precursors in addition to PIs as suggested by Boccino et al. (18). In the present study, long-term incubation with PMA produced a persistent elevation of DAG levels comparable to that produced by S^1-All. These persistent elevations of DAG were associated with increased choline release, suggesting that PC hydrolysis was the mechanism by which delayed DAG production occured. The dose of 0.1 μM of PMA activates PKC, but does not fully down regulate the enzyme (2). If a high concentration of PMA (2 μM) was used, no increase in DAG-levels could be observed at 18 hrs (unpublished observation). Therefore, continuous PKC activation caused either directly by PMA or indirectly through All receptor activation of PLC appears necessary for long-term DAG production. Activated PKC may thus not only inhibit DAG production from PIs, but also stimulate production of DAG from PC.

In many cells, PKC activation increases DAG which is derived from PC hydrolysis (23, 24). In the present study both S^1-All and PMA significantly increased the release of (^{3}H)choline after 60 min of incubation. Less pronounced increases were observed in some experiments at 3 and 12 min, but not after 18 hours at which time the releasable PC pool may have been depleted. These findings suggest that the All-induced sustained DAG production in BAM cells resulted from PKC dependent PC hydrolysis. However, these results do not define the mechanism for PC hydrolysis, which may depend on PLD- or PLC-activation (25).

In summary, continuous incubation with either S^1-All or PMA persistently increase DAG levels in BAM cells. The initial peak in DAG levels may be derived from the breakdown of phosphoinositides. The later increases apparently are derived from breakdown of PC. These results add to the growing body of evidence (19, 26, 27) that DAG production can occur in more than one phase. These results also support our earlier results on the important role of PKC in mediating the long-term effects of All on gene expression in BAM cells (1,2).

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