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PHOSPHOLIPASE A_2-ACTIVATING PEPTIDE-INDUCED CONTRACTION OF SMOOTH MUSCLE IS MEDIATED BY PROTEIN KINASE C - MAP KINASE CASCADE

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The mammalian phospholipase A_2-activating protein (PLAP) affects contraction of smooth muscle cells isolated from the rabbit rectosigmoid. PLAP (10^{-6} M)-induced contraction peaked at 30 sec and was sustained at 4 min. MAP kinase was activated by PLAP (10^{-6} M), as measured using myelin basic protein (MBP) as substrate. The increase in MAP kinase activity was rapid at 30 sec (159±2.5%) and remained at a sustained level (162±7.9%) at 4 min. Preincubation of the cells with the PLA_2 inhibitor ONO-RS-082 (10^{-6} M) or with the PKC inhibitor calphostin C (10^{-6} M) resulted in inhibition of contraction, as well as inhibition of the associated increase in MAP kinase activation. The data indicates that PLAP-specific contractile effect on isolated smooth muscle cells is mediated by an activation of a PKC-MAP kinase cascade and suggests a putative role for PLA_2-coupled G protein activation of PKC-MAP kinase as an alternate transduction pathway in smooth muscle contraction.

Phospholipase A_2(PLA_2) catalyzes the hydrolysis of the ester linkage at the sn-2 position of phospholipids to produce free fatty acids and lysophospholipids and a series of functionally significant eicosanoids that participate in signal transduction in several tissues [1][2]. Most mammalian cells have at least two types of PLA_2: a secreted form ($sPLA_2$) and a high molecular-mass(85-110 kDa) cytosolic form ($cPLA_2$) [3] found in numerous tissues, including platelets [4][5][6], renal mesangial cells, and kidney [7]. The 749-residue sequence of $cPLA_2$ has no homology to $sPLA_2$. Ca^{++} binding and phosphorylation, enhance translocation of $cPLA_2$ to the membrane for interaction with its substrates [8]. Evidence indicates that $cPLA_2$ is involved in the signal transduction process [9][10]. In esophageal muscle cells, a 100 kDa $cPLA_2$ participates in acetylcholine-induced smooth muscle contraction by producing arachidonic acid and potentiating DAG-induced activation of a PKC-dependent pathway [11].

A mammalian protein which activates PLA_2 has been cloned [12]. It shares antigenic and biochemical similarities with melittin, a well characterized bee venom phospholipase-stimulatory peptide [13]. Using this phospholipase A_2 activating peptide (PLAP), we have examined the

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Abbreviations: DAG, diacylglycerol; EGTA, ethyleneglycol-bistetraacetic acid; kDa, kilo-Dalton; MAP Kinase, Mitogen Activated Protein Kinase; PLAP, Phospholipase A2 Activating Peptide; PKC, protein kinase C.

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mechanism by which PLA_2 affects contraction of smooth muscle cells isolated from the rabbit rectosigmoid.

Materials and Methods

Materials: The following reagents were purchased. PLAP and ONO-RS-082, ketoconazole, nordihydroguaiaretic acid (NDGA) and indomethacin were purchased from Biomol (Plymouth Meeting, PA). Monoclonal mouse anti-MAP kinase (ERK1+2) antibody was from Zymed Laboratories Inc.(South San Francisco, CA). Collagenase type II was purchased from Worthington biochemical corporation (Freehold, NJ). Myelin Basic Protein (MBP) from bovine brain was purchased from Sigma (St. Louis, MO), Calphostin-C was from Kamiya Biomedical Company (Thousand Oaks, CA). [γ-^{32}P]ATP and enhanced chemiluminescence (ECL) detection reagents were obtained from Amersham (Arlington Heights, IL). All other reagents were purchased from Sigma.

Isolation of smooth muscle cells from rabbit rectosigmoid: The internal anal sphincter, consisting of the distal most 3 mm of the circular muscle layer, ending at the junction of skin and mucosa, was removed by sharp dissection. A 5 cm length of the rectosigmoid orad to the junction was dissected and digested to yield isolated smooth muscle cells. Cells were isolated by enzyme digestion with collagenase as previously described [14]. Each rectosigmoid yielded 10-20 x 10^6 cells.

Preparation of permeable smooth muscle cells: In experiments involving preincubation of smooth muscle cells with monoclonal antibodies which do not diffuse across the intact cell membrane, muscle cells are made permeable as described in our previous report. [14]. Isolated cells are permeabilized by incubation for 3 min in saponin (75 μg/ml). The cell suspension is then washed in cytosolic buffer and resuspended in the cytosolic buffer containing antimycin A (10 μM), ATP (1.5 mM), and an ATP-regenerating system consisting of creatine phosphate (5 μM) and creatine phosphokinase (10 units/ml).

Measurement of contraction: Muscle cells are examined within 30 min of dispersion. Aliquots consisting of 2.5 x 10^4 cells in 0.5 ml or medium are added to 0.1 ml of a solution containing PLAP. When inhibitors were used, the cells were incubated in appropriate concentrations of the inhibitors for 20 min before stimulation with PLAP. When anti-MAP kinase antibody was used, permeabilable cells were incubated in the antibody at a 1:100 dilution for 20 min before stimulation. The reaction is interrupted at indicated intervals (30sec and 4min) by the addition of 0.1 ml of acrolein at a final concentration of 1%. Individual cell length is measured by computerized image micrometry. The average length of cells in the control state or after addition of test agents is obtained from 50 cells encountered randomly in successive microscopic fields. The contractile response is defined as the decrease in the average length of the 50 cells and is expressed as the absolute change or the percent change from control length [14].

Immunoblotting of MAP kinase using monoclonal anti-MAP kinase antibody: Cells were treated with PLAP for indicated periods of time and immunoblotting was performed as previously described[15]. In brief, particulate fractions (50μg-protein) were subjected to SDS-PAGE (12%) and electrophoretically transferred to PVDF membrane. Immunoblotting was performed using mouse monoclonal anti-MAP kinase antibody (Anti-ERK1+2) (1:2000 dilution) as a primary antibody. Then the membrane was reacted with peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution) for 1 hour. The enzymes on the membrane were visualized with chemiluminescent substrates (ECL; Amersham, IL).

Assay of MBP kinase in polyacrylamide gel copolymerized with MBP: In order to confirm that MBP is the substrate for MAP kinase activation, MAP kinase activity was also determined in polyacrylamide gels according to the method of Kameshita and Fujisawa [16] with slight modifications [17]. Cell extracts (100μg protein) were resolved on a 10% SDS-polyacrylamide gel copolymerized with 0.5mg/ml MBP. After electrophoresis, gels were washed with four changes of 50mM Tris, pH 8.0, containing 20% propanol to remove SDS, and then with two changes of denaturing buffer containing 6M guanidine hydrochloride, 50mM Tris, and 5mM mercaptoethanol. The enzymes on the gel were renatured with four changes of 250ml renaturing buffer containing 50mM Tris, 0.04% (v/v) Tween 40, and 5mM mercaptoethanol at 4°C for 21h. The gel was incubated with assay buffer containing 40mM HEPES, pH 8.0, 10mM $MgCl_2$, 2mM DTT, and 0.1mM EGTA at 30°C for 30min. The kinase activity was determined by incubating the gel with 40ml of the assay buffer plus 20μM ATP and 100μCi [γ-^{32}P]ATP at 30°C for 1h. After the incubation, the gel was extensively washed nine times for 10min each with 250ml 5%

trichloroacetic acid and 10mM sodium pyrophosphate solution to eliminate nonspecific radioactivity in the gel. The gel was then dried and subjected to autoradiography on Kodak X-Omat AR film at -80°C.

Time course of MAP kinase activity stimulated by PLAP: MAP kinase activity was assayed using myelin basic protein (MBP) as a substrate [17]. The cell lysates were prepared as described above. 10μl of each sample was incubated in 15μl of assay buffer (final concentrations: 0.6mg/ml MBP, 60μ M ATP, 16mM $MgCl_2$, 1.2mM EGTA, 2.5 μ Ci [γ-^{32}P]ATP, 0.15M tris (hydroxymethyl) aminomethane (Tris), pH 7.4) for 15 min. 15μl of the reacted mixture was spotted onto Whatman P-81 paper and washed 4 times thoroughly in 250ml of 1% phosphoric acid, followed by two additional washes with 100ml of absolute ethanol. Finally, to determine incorporated [γ-^{32}P]-labeled phosphate into MBP, the radioactivity of the each paper was counted by liquid scintillation counting, and the amount of phosphorylated MBP was calculated. The magnitude of the activation was expressed as percent ratio of each activity to the initial activity. Points and bars represent mean ± SE of 3-4 experiments.

Statistical analysis: Data are represented as mean ± SE and were obtained from two to four separate experiments. The significance was evaluated by unpaired Student's *t* -test.

Results

PLAP-induced contraction of smooth muscle cells: PLAP (10^{-6}M) induced a maximal contractile effect on smooth muscle cells isolated from the rabbit rectosigmoid. As shown in Figure 1, the percent decrease in cell length peaked at 30 seconds (33.9±1.5%), and remained sustained at 4 min after stimulation (30.8±4.1% decrease in cell length from control). Preincubation of the cells with the PLA_2 specific inhibitor ONO-RS-082 (10^{-6}M), for 20min caused a total inhibition of PLAP-induced contraction, indicating the involvement of PLA_2 pathways in the contractile effect of PLAP.

In order to study the involvement of PKC in PLAP-induced contraction, we assessed the effect of the PKC inhibitor, calphostin C on PLAP-induced contraction. Preincubation of the cells with calphostin C(10^{-6}M) for 20min totally inhibited PLAP-induced contraction. On the other hand, preincubation of the cells with inhibitors of lipoxygenase (NDGA, 10^{-5}M), of cytochrome P-450 (ketoconazole, 10^{-5}M) and of cyclooxygenase (indomethacin, 10^{-5}M), had no effect on the action of PLAP, suggesting that PLAP-induced contraction is mediated through a PKC dependent pathway (Fig 1).

Activation of MAP kinase by PLAP: To determine whether the contractile effect of PLAP is related to the activation of MAP kinase in isolated smooth muscle cells of the rabbit rectosigmoid, cells were stimulated with PLAP (10^{-6}M) for different periods of time and immunodetection of MAP kinase in cell lysates followed by substrate phosphorylation assay was performed.

We have previously reported the localization of MAP kinase in either resting or contracted smooth muscle cells [15]. In the resting cell, MAP kinase is distributed throughout the cell along the membrane. In response to bombesin-induced contraction, MAP kinase translocated into a general distribution throughout the cytoplasm. Furthermore, immunodetection of MAP kinase in either cytosolic and particulate fraction of the cell indicated that, after stimulation with bombesin, immunoreactivity to MAP kinase antibody decreased and disappeared in the particulate fraction, while it increased in the cytosolic fraction. In response to PLAP (10^{-6}M), a similar increase in MAP kinase activity was observed associated with a decrease in the intensity of the band identified as MAP kinase p42 in the particulate fraction (Fig 2).

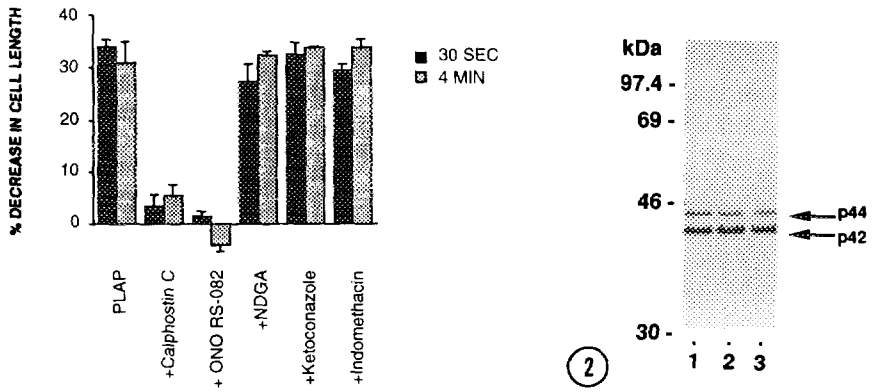

Figure 1. Inhibition of PLAP-induced contraction by Calphostin C, ONO-RS-082, NDGA, ketoconazole and indomethacin. Freshly isolated cells were pretreated with various inhibitors for 20min prior to stimulation with PLAP(10^{-6}M). Preincubation with ONO-RS-082(10^{-6}M) or Calphostin C(10^{-6}M) totally inhibited PLAP peptide-induced contraction. On the other hand, inhibitors of lipoxygenase (nordihydroguaiaretic acid; NDGA, 10^{-5} M), cytochrome P-450 (ketoconazole, 10^{-5}M) and cyclooxygenase (indomethacin, 10^{-5}M) had no effect on the action of PLAP peptide.

Figure 2. Western blot analysis in particulate fractions. Highly fractionated insoluble particulate fractions were prepared from relaxed freshly isolated smooth muscle cells (lane 1), and from cells that were stimulated with PLAP 10^{-6}M (lane 2, 30sec and lane 3, 4min after stimulation). The figure shows that the intensity of the bands identified as MAP kinase p44 decreased in the particulate fraction after stimulation with PLAP.

To determine whether the PKC-mediated contractile effect of PLAP is related to the activation of MAP kinase, cells were stimulated with PLAP (10^{-6}M) for different periods of time and substrate phosphorylation assay was performed. Treatment of the cells with PLAP (10^{-6}M) caused a 1.6 fold increase in MAP kinase activity assayed in the lysates (Fig. 3). The time course for activation of MAP kinase showed a rapid increase at 30 seconds (159±2.5%, $p < 0.0005$, n=4) and remained sustained at 4 minutes (162±7.9%, $p < 0.0005$, n=4). (Fig.3 open circles). Preincubation of cells with a PLA_2 inhibitor ONO-RS-082 (10^{-6}M) [18], totally inhibited MAP kinase activation induced by PLAP. (Fig.3 closed triangles). As shown in Fig.4, the MAP kinase activity stimulated by PLAP was demonstrated in MBP-copolymerized polyacrylamide gel whereby a 44 kDa protein was enhanced by stimulation with PLAP (10^{-6}M).

Role of PKC in PLAP-induced MAP kinase activation: We then investigated whether PKC activation is involved in the PLAP-induced activation of MAP kinase. Calphostin C is known to interfere with diacylglycerol binding to the regulatory domain of PKC [19]. Preincubation of smooth muscle cells with calphostin C (10^{-6}M), did not alter basal MAP kinase activity (basal MAP kinase activity of Calphostin C-pretreated cells was 30.3 ± 2.9 pmol/min/mg-protein vs. 29.7± 2.9 pmol/min/mg-protein in control cells, $p > 0.4$, Non significant: Student's *t* test). As shown in Fig 3(closed squares), Calphostin C totally inhibited PLAP-induced MAP kinase activation. These observations leads us to suggest that activation induced by PLAP may involve a cascade of events involving first an activation of PKC and followed by activation and possible redistribution of MAP kinase.

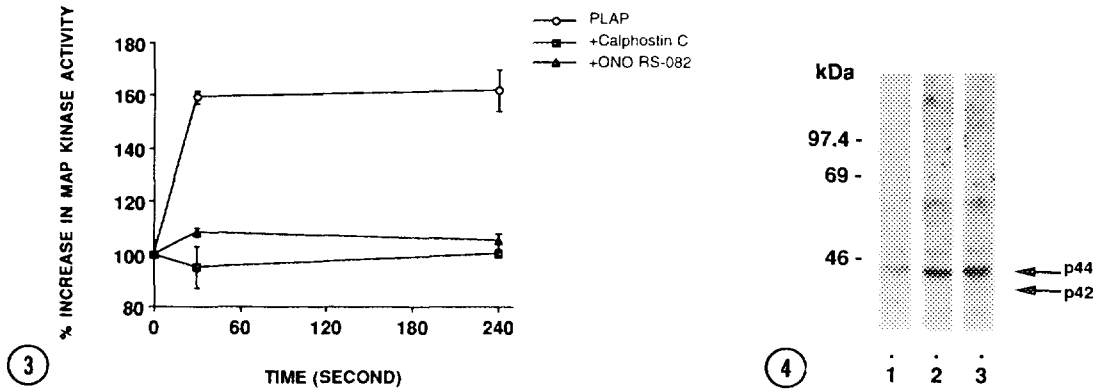

Figure 3. Time course of MAP kinase activity stimulated by PLAP. MAP kinase activity was assayed using myelin basic protein (MBP) as a substrate. Activity increased rapidly at 30 sec and was sustained at 4 minutes.(open circles). Preincubation of cells with Calphostin C (closed squares) or ONO-RS-082(closed triangle) totally inhibited MAP kinase activation. (Basal MAP kinase activity was 30.6±3.6 pmol/min/mg-protein, n=8) Points and bars represent mean±SE of 3-4 experiments. The mean counts per minute values of control, PLAP stimulated, and buffer alone in one experiment were 19126.9, 31731.9, 646.5, respectively.

Figure 4. Determination of MBP kinase activity in polyacrylamide gel copolymerized with MBP. In order to confirm that MBP is the substrate for MAP kinase activation, we have performed a kinase assay in polyacrylamide gel containing 0.5 mg/ml MBP. Cell lysates were subjected to electrophoresis in 10% polyacrlamide gel containing 0.5mg/ml of MBP. The enzymes resolved in the gel were exposed to denaturing and renaturing procedures and were then incubated in a solution containing 2.5μCi/ml [g-^{32}P]ATP. The phosphorylated bands were visualized by autoradiography. Several proteins with the ability to phosphorylate MBP were detected (lane 1), but the band estimated as 44kDa was dominantly enhanced by stimulation with PLAP (lane 2, 30sec and lane 3, 4min after stimulation). The incorporation of ^{32}P by the 42-kDa protein was much less than that by 44-kDa protein.

Role of MAP kinase in PLAP-induced contraction: To ascertain whether MAP kinase indeed performs a functional role in PLAP-induced contraction of smooth muscle cells, we further studied the effect of pretreatment with anti-MAP kinase antibody prior to stimulation with PLAP. In control experiments, preincubation of smooth muscle cells with control non-immune IgG did not affect the length of cells in the control state and had no effect on PLAP-induced contraction. As shown in Fig.5, preincubation of permeabilized smooth muscle cells with MAP kinase antibody (1:100) for 20 min inhibited (83.9±2.4% inhibition, $p < 0.0005$, n=3) the contraction induced by PLAP.

Discussion

PLAP was originally identified on the basis of its immunological cross-reactivity with antibodies to bee venom PLA_2 stimulating peptide, melittin [20]. PLAP was cloned and increased expression of PLAP mRNA by treatment with LTD4 was also demonstrated [21]. The relevance of this protein in the activation of PLA_2 had been confirmed in experiments using synthetic antisense DNA in smooth muscle and endothelial cells [21]. Upon activation of PLA_2, PLA_2 is capable of metabolizing membrane phospholipids, which result in the production of aracidonic acid and lysophospholipids. PLA_2 activated by PLAP is thought to be the high molecular weight, cytosolic type because of its selectivity to produce the eicosanoid [21]. This enzyme contains a

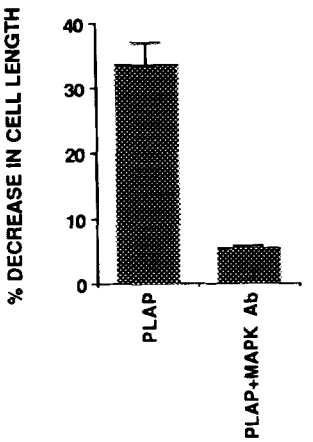

Figure 5. Inhibition of PLAP-induced contraction by monoclonal anti-MAP kinase antibody. Permeabilized cells were incubated with anti-MAP kinase antibody (1:100) for 20min. before stimulation with PLAP (10^{-6} M) for 30s. The contractile response was determined as the decrease in average length of 50 cells and expressed as the percent change from control length. Pretreatment with MAP kinase antibody totally inhibited the initial transient contraction at 30sec. Data are expressed as mean ± SE of 3 experiments.

Ca^{++}/phospholipid binding domain analogous to C2 domain of Ca^{++}-dependent PKC [21], and it is thought to be sensitive to G-proteins and PKC in many cell types [22][21][23][24].

Our present data indicate that PLAP induces contraction of smooth muscle cells isolated from rabbit rectosigmoid colon. While the PLA_2 inhibitor, ONO-RS-082, specifically inhibited contraction by PLAP, the inhibitors of lipoxygenase, of cytochrome P-450 and of cyclooxygenase had no effect on PLAP-induced contraction. Our data thus suggest that arachidonic acid, but not arachidonic acid metabolites, may function as a signal messenger to induce smooth muscle cell contraction. In guinea pig intestinal smooth muscle cells, it has been reported that arachidonic acid released after agonist-induced G protein-dependent transient activation of PLA_2 elicits contraction and Ca^{++} influx which subsequently triggers Ca^{++}-induced Ca^{++} release [25], and in esophageal muscle cells, a 100 kDa $cPLA_2$ was found to participate in acetylcholine-induced smooth muscle contraction by producing arachidonic acid and potentiating DAG-induced activation of PKC-dependent pathway [11].

Mitogen-activated protein(MAP) kinase is a serine/threonine specific protein kinase [26], the activation of which is induced by a variety of extracellular stimuli. Smooth muscle cells isolated from rabbit rectosigmoid contain 42- and 44-kDa MAP kinases, which are activated by stimulation with bombesin [15] or sphingosylphosphorylcholine(SPC) [17] through a PKC-dependent pathway. In the present study, we have investigated MAP kinase activation in response to PLAP-induced contraction. Using monoclonal anti-MAP kinase antibody, our data indicate the presence of two forms of MAP kinase(44- and 42-kDa) by immunoblotting. In the particulate fractions, the immunoreactivity to MAP kinase antibody significantly decreased upon stimulation with PLAP, suggesting translocation of MAP kinase to the cytosolic fraction. In the kinase assay, the activity of MAP kinase rapidly increased at 30 sec after PLAP-stimulation and remained sustained at 4 min. MAP kinase activation was totally blocked by pretreatment of the cells with the PLAP inhibitor

ONO-RS-082, indicating the specificity of PLAP-induced contraction and PLAP-induced activation of MAP kinase. MAP kinase activation by PLAP was also inhibited by preincubation of the cells with the PKC inhibitor, Calphostin C, indicating that PLAP-induced activation of MAP kinase is mediated through PKC. We have previously shown that direct activation of PKC by TPA(10^{-6}M) resulted in a potent increase in MAP kinase activity [15], which was inhibited by pretreatment with calphostin C, indicating that MAP kinase activation is mediated by PKC. Taken together, these data lead us to suggest that signaling pathway initiated by PLAP activation is through PKC activation of MAP kinase leading to contraction. Preincubation of permeabilized cells with MAP kinase antibody inhibited PLAP-induced contraction, indicating that MAP kinase has a functional role in PLAP-induced contraction.

PLAP has a sequence homology with G_β subunit and has been identified as a new member of the G_β superfamily [27]. Tsunoda, et al.[28][29] suggested that G protein coupled to PLA_2 may be similar or identical to G_β or $G_{\beta/\gamma}$ in pancreatic acinar cells. PLA_2 activation by PLAP, elicited Ca^{++} oscillations and monophasic amylase secretion via the arachidonic acid pathway. In COS-7 cells, Gi-coupled receptor stimulation leading to MAP kinase activation is mediated primarily through $G_{\beta/\gamma}i$ [30][31]. The cellular over expression of β–adrenergic receptor kinase(βARK)ct has provided evidence that Gi-mediated MAP kinase activation involves $G_{\beta/\gamma}i$-dependent activation of Ras [32]. The data suggest that in smooth muscle cells, PLAP, which has a sequence homology with G_β subunit, induces contraction and activates MAP kinase through a pathway similar to $G_{\beta/\gamma}$. Since the biochemical mechanisms by which PLAP induces PLA_2 activation have not been well characterized, it is difficult to conclude where PKC is implicated in PLAP-induced contraction. Two alternatives are possible: One is upstream of PLA_2 activation and the other is between PLA_2 activation and MAP kinase activation. Preincubation of the cells with the PLA_2 inhibitor ONO-RS-082 resulted in inhibition of MAP kinase activation. The data thus suggest that PLAP activation of MAP kinase is dependent on PLA_2 activation. Thus, PLAP might directly activate PLA_2 which leads to activation of PKC-MAP kinase-contraction cascade; or PLAP activates PKC-MAP kinase cascade by direct activation of PLA_2.

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