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Long-term potentiation requires activation of calcium-independent phospholipase A_2

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Abstract The predominant phospholipase activity present in rat hippocampus is a calcium-independent phospholipase A_2 (302.9 ± 19.8 pmol/mg·min for calcium-independent phospholipase A_2 activity vs. 14.6 ± 1.0 pmol/mg·min for calcium-dependent phospholipase A_2 activity). This calcium-independent phospholipase A_2 is exquisitely sensitive to inhibition by the mechanism-based inhibitor, (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyran -2-one (BEL). Moreover, treatment of hippocampal slices with BEL prior to tetanic stimulation prevents the induction of LTP (40.8 ± 5.6% increase in excitatory post-synaptic potential (EPSP) slope for control slices ($n = 6$) vs. 5.8 ± 8.5% increase in EPSP slope for BEL-treated slices ($n = 8$)). Importantly, LTP can be induced following mechanism-based inhibition of phospholipase A_2 by providing the end product of the phospholipase A_2 reaction, arachidonic acid, during the application of tetanic stimulation. Furthermore, the induction of LTP after treatment with BEL is dependent on the stereoelectronic configuration of the fatty acid provided since eicosa-5,8,11-trienoic acid, but not eicosa-8,11,14-trienoic acid, rescues LTP after BEL treatment (37.6 ± 16.1% increase in EPSP slope for eicosa-5,8,11-trienoic acid vs. −3.7 ± 5.2% increase in EPSP slope for eicosa-8,11,14-trienoic acid).Collectively, these results provide the first demonstration of the essential role of calcium-independent phospholipase A_2 in synaptic plasticity.

Key words: Phospholipase A_2; Arachidomic acid; LTP; Hippocampus

1. Introduction

Long-term potentiation (LTP) is an electrophysiologic property of organized neural structures that has been extensively utilized as a paradigm for the investigation of the biochemical pathways underlying synaptic plasticity and memory [1–3]. The study of hippocampal LTP has been a preferred experimental model due to the planar arrangement of the Schaffer collateral pathways leading to the CA1 region of the hippocampus. The induction of LTP in the CA1 region of the hippocampus typically involves the activation of NMDA and metabotropic glutamate receptors, which increase intracellular calcium concentration [3–6]. Stimulation of these glutamate receptors promotes the activation of phospholipase A_2 leading to the release of arachidonic acid [7,8] and the initiation of PAF synthesis. Since both eicosanoids and PAF facilitate excitatory synaptic transmission and LTP [9–13], these studies have collectively suggested a critical link between phospholipase A_2 activation and the induction of LTP.

Due to the importance of calcium ion in mediating LTP, it has been traditionally assumed that the enzyme mediating the release of arachidonic acid during LTP was a calcium-dependent phospholipase A_2 whose activation paralleled glutamate receptor mediated alterations in intracellular calcium ion concentration [14,15]. Since both arachidonic acid and PAF have been implicated in LTP, and since calcium-independent phospholipase A_2 is selective for the hydrolysis of ether-linked phospholipids [16] (which are the predominant storage depot of arachidonic acid in brain [17]), we exploited the specificity inherent in mechanism-based inhibition to investigate the role of calcium-independent phospholipase A_2 in the biochemical events responsible for LTP in the hippocampus. We now report that calcium-independent phospholipase A_2 is the predominant phospholipase A_2 in the hippocampus and that calcium-independent phospholipase A_2 mediates the release of arachidonic acid during LTP.

2. Experimental procedures

2.1. Materials

Radiolabeled 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-^{3}H] octadec-9′-enoyl-*sn*-glycero-3-phosphocholine and the calcium-independent phospholipase A_2 inhibitor, BEL, were synthesized by established methods [18–20]. Recombinant 85 kDa $cPLA_2$ (GenBank Accession #M68874) was purified from baculovirus infected Sf9 insect cells by FPLC Mono-Q chromatography. The specific activity of recombinant $cPLA_2$ before addition to hippocampal homogenates (~1 μmol/mg·min) and that attributable to exogenous $cPLA_2$ were similar to that previously reported [21,22].

2.2. Quantification of calcium-independent phospholipase A_2

Rat hippocampi and whole brains (postnatal day 30) were rapidly excised and homogenized in buffer (340 mM sucrose, 10 mM HEPES, 1 mM EDTA, 0.1 mM DTT, pH 7.5). Homogenates were centrifuged

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Abbreviations: AACOCF3: 1,1,1-trifluoromethyl-eicosa-5,8,11,14-tetraenone; BEL, (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyran -2-one; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; $cPLA_2$, calcium-dependent phospholipase A_2; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EPSP, excitatory post-synaptic potential; LTP, long-term potentiation; NDGA, nordihydroguiaretic acid; NMDA, *N*-methyl-D-aspartate; PAF, platelet activating factor; PS, population spike.

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at 100,000×g_{max} for 60 min and the resultant supernatant (cytosol) was removed and utilized within 2 h. Phospholipase A_2 activity was quantified by measuring the release of radiolabeled fatty acid from synthetically prepared 1-*O*-(Z)-hexadec-1′-enyl-2-[9,10-^{3}H]octadec-9′enoyl-*sn*-glycero-3-phosphocholine in buffer (100 mM Tris-HCl, pH 7.0/37°C)containing either 10 mM EGTA, 1 mM $CaCl_2$, or 10 mM $CaCl_2$ [16,23]. Reactions were typically conducted with 5 μM radiolabeled glycerophospholipid substrate and 50 μg of cytosolic protein in a final assay volume of 210 μl. After incubation at 37°C for 2 min, reactions were quenched by the addition of 100 μl of *n*-butanol, vortexed, phases were separated by centrifugation and the reaction products were resolved by thin layer chromatography (Whatman silica gel 60A plates) utilizing a mobile phase comprised of 80:20:1 (petroleum ether/diethyl ether/acetic acid (v:v)) and radioactivity in fatty acids was quantified [16].

2.3. Electrophysiology

Male albino rats, 28–32 days old, were anesthetized with halothane, sacrificed, and hippocampi were dissected and placed in standard buffer containing (in mM): NaCl 124, KCl 5, $CaCl_2$ 2, $NaHCO_3$ 22, NaH_2PO_4 1.25, $MgSO_4$ 2, and glucose 10 (pH 7.4, 4°C; 95% O_2/CO_2). Transverse slices (500 μm thick) were cut from the septal half of the hippocampus utilizing a Campden vibratome and maintained in standard buffer at 30°C for at least one hour. For physiologic studies, slices were submerged in a constant flow (2 ml/min) recording chamber and maintained at 30°C for the course of the experiment. Extracellular recordings were obtained from the dendritic layer of the CA1 region using 5–10 MΩ broken tip glass electrodes filled with 2 M NaCl. Evoked synaptic responses were elicited with 0.2 ms constant current pulses through a bipolar electrode placed in the Schaffer collateral–commisural pathway. EPSPs were monitored by applying single pulses every 1 min at an intensity sufficient to evoke a 50% response of the maximal PS amplitude. LTP was produced by a 100 Hz electrical tetanus for 1 s. Potentiation was measured as the change in the EPSP slope or PS amplitude at the 50% point on the respective input-output curves at 60 min post-tetanus [24]. BEL, $AACOCF_3$, arachidonic acid, eicosa-5,8,11-trienoic acid, and eicosa-8,11,14-trienoic acid were administered in standard buffer solutions into the recording chambers though individual tubes.

3. Results and discussion

Phospholipase A_2 activity in rat hippocampal homogenates was predominantly calcium-independent (the specific activity of calcium-independent phospholipase A_2 was 302.9 ± 19.8 pmol/mg·min vs. 14.6 ± 1.0 pmol/mg·min for calcium-dependent phospholipase A_2; Fig. 1A) and over 95% of hippocampal phospholipase A_2 activity was inhibited by the mechanism-based inhibitor BEL (10 μM) which possesses over a 1000-fold selectivity for the inhibition of calcium-independent phospholipase A_2 in comparison to calcium-dependent phospholipases A_2 [18,19]. Remarkably, rat hippocampus was 7-fold enriched in calcium-independent phospholipase A_2 activity compared to phospholipase A_2 activity manifest in homogenates of whole brain (Fig. 1A).

To further substantiate the dearth of calcium-dependent phospholipase A_2 activity present in the hippocampus, we demonstrated: (1) the absence of calcium-mediated augmentation of phospholipase A_2 activity in the absence or presence of BEL (Fig. 1A); and (2) the absence of detectable immunoreactive 85 kDa calcium-dependent phospholipase A_2 by Western blotting (data not shown). Furthermore, when highly purified recombinant 85 kDa $cPLA_2$ was added to crude hippocampal homogenates prior to the preparation of cytosol and microsomes (to ensure that the homogenization conditions employed did not inactivate calcium-dependent phospholipase A_2 activity) recov-

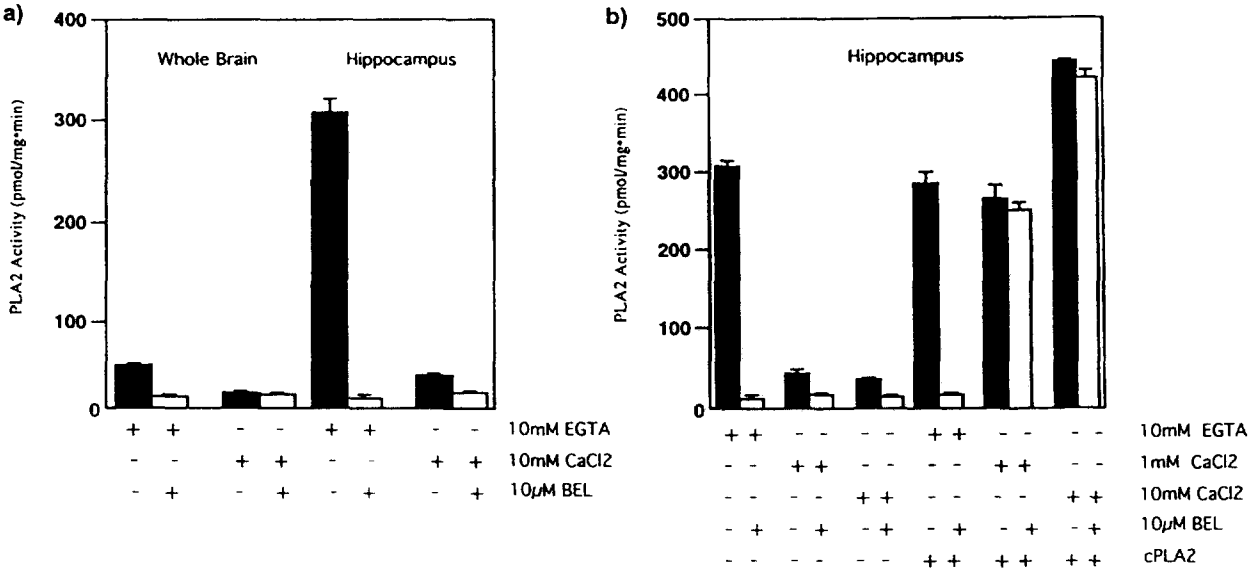

Fig. 1. The predominance of calcium-independent phospholipase A_2 activity in rat hippocampus and its specific mechanism-based inhibition by (*E*)-6-(bromomethylene)-3-(1-naphthal-enyl)-2*H*-tetrahydropyran-2-one (BEL). (A) Phospholipase A_2 activity in homogenates of either rat whole brain or hippocampus was quantified by measuring the release of radiolabeled fatty acid from choline glycerophospholipids in the absence or presence of calcium ion as described in section 2. The susceptibility of calcium-dependent and calcium-independent phospholipase A_2 activity to inhibition by BEL was examined by preincubation of protein with either vehicle alone (solid bars) or BEL (10 μM) (open bars) prior to incubation with *sn-2* radiolabeled choline glycerophospholipids in the absence or presence of calcium ion. Values represent the mean ± S.E.M. (n = 3). (B) To substantiate the predominance of calcium-independent phospholipase A_2 activity in rat hippocampus demonstrated in panel A, purified recombinant 85 kDa $cPLA_2$ was added to hippocampal homogenates and the recovery of exogenously added $cPLA_2$ as well as its susceptibility to mechanism-based inhibition by BEL was assessed. Addition of recombinant $cPLA_2$ (~5 μg) resulted in the recovery of over 90% of added $cPLA_2$ activity and recovered activity was not susceptable to inhibition by BEL. Approximately 95% of the exogenously added $cPLA_2$ activity partitioned with the cytosol (data not shown). Values represent the mean ± S.E.M. (n = 3).

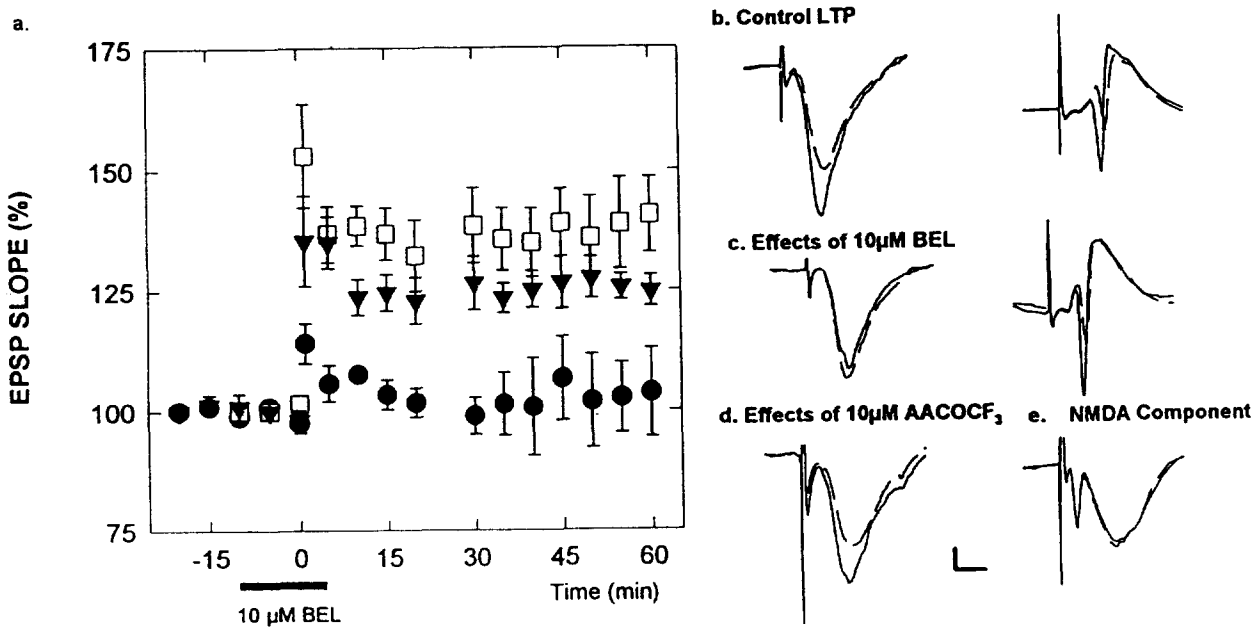

Fig. 2. BEL inhibits the induction of LTP in the CA1 region of the hippocampus. (A) The time course of the change in EPSP slope (mean ± S.E.M.) was determined for a series of control slices (open squares), slices treated with 10 μM $AACOCF_3$ (filled triangles) or 10 μM BEL (filled circles) as described in section 2. The phospholipase A_2 inhibitors, $AACOCF_3$ or BEL, were delivered 10 min before and 5 min after the tetanus at time zero (solid bar). (B,C) The traces depict field EPSPs (left) and population spikes (right) in representitive experiments measured before (dashed line) and 60 min after (solid line) the tetanus in control slices (B) and slices treated with either 10 μM BEL (C) or 10 μM $AACOCF_3$ (D). The trace in (E) depicts the effect of 10 μM BEL on the NMDA component of synaptic responses recorded in the presence of 25 μM CNQX and 0.1 mM Mg^{2+}. Calibration bar = 1 mV, 5 ms.

ered recombinant $cPLA_2$ had a specific activity and calcium sensitivity indistinguishable from the enzyme purified from natural sources [21,22] (Fig. 1B). The ability of BEL to discriminate between these two classes of intracellular phospholipases A_2 is underscored by the complete ablation of calcium-independent phospholipase A_2 activity by BEL (309 ± 12.8 pmol/mg·min in the absence of BEL vs. 13.0 ± 4.2 pmol/mg·min in the presence of BEL (10 μM)) while calcium-dependent phospholipase A_2 activity was not inhibited by BEL (445.0 ± 3.0 pmol/mg·min in the absence of BEL vs. 423.5 ± 7.9 pmol/mg·min in the presence of BEL (10 μM)).

We exploited the specificity inherent in mechanism-based

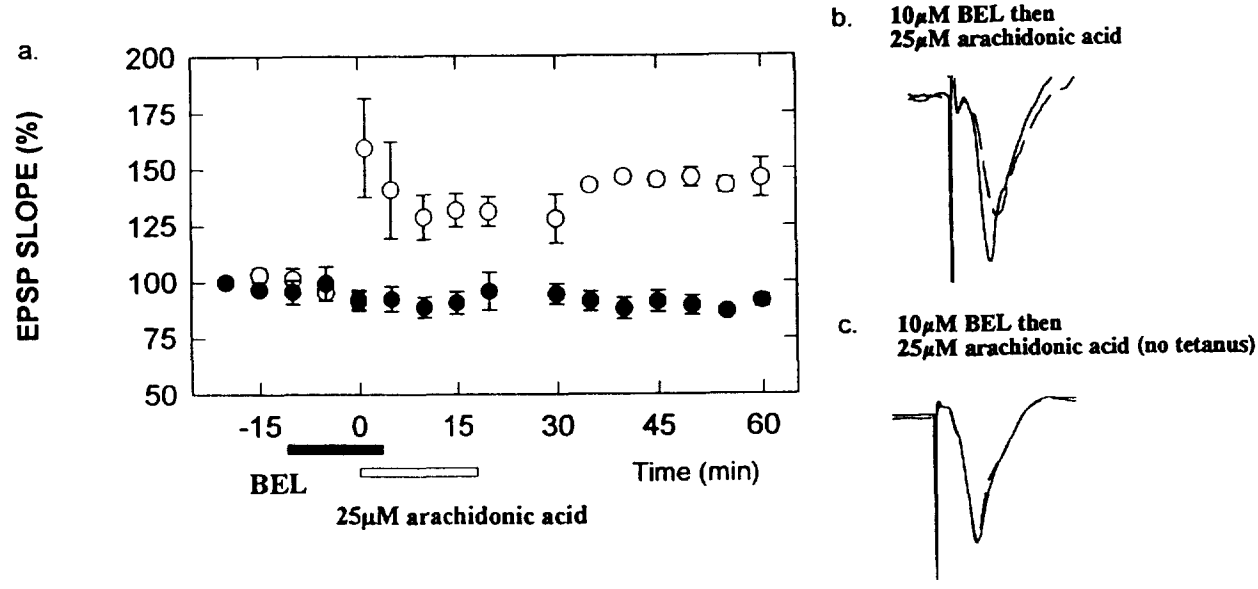

Fig. 3. Arachidonic acid abolishes the BEL-mediated inhibition of LTP in the hippocampus. (A) The time course of the change in EPSP slope (mean ± S.E.M.) was determined for slices treated with 10 μM BEL (filled circles) or with 10 μM BEL followed by the application of 25 μM arachidonic acid (open circles). BEL was administered for 10 min before and 5 min after the tetanus (time zero) (solid bar) and arachidonic acid was applied immediately after the tetanus (open bar). (B) The trace depicts a representative EPSP before (dashed line) and 60 min after (solid line) the tetanus in BEL-treated slices with provision of 25 μM arachidonic acid. (C) The trace depicts a representative EPSP before (dashed line) and 60 min after (solid line) the incubation of BEL and application of 25 μM arachidonic acid without the tetanus.

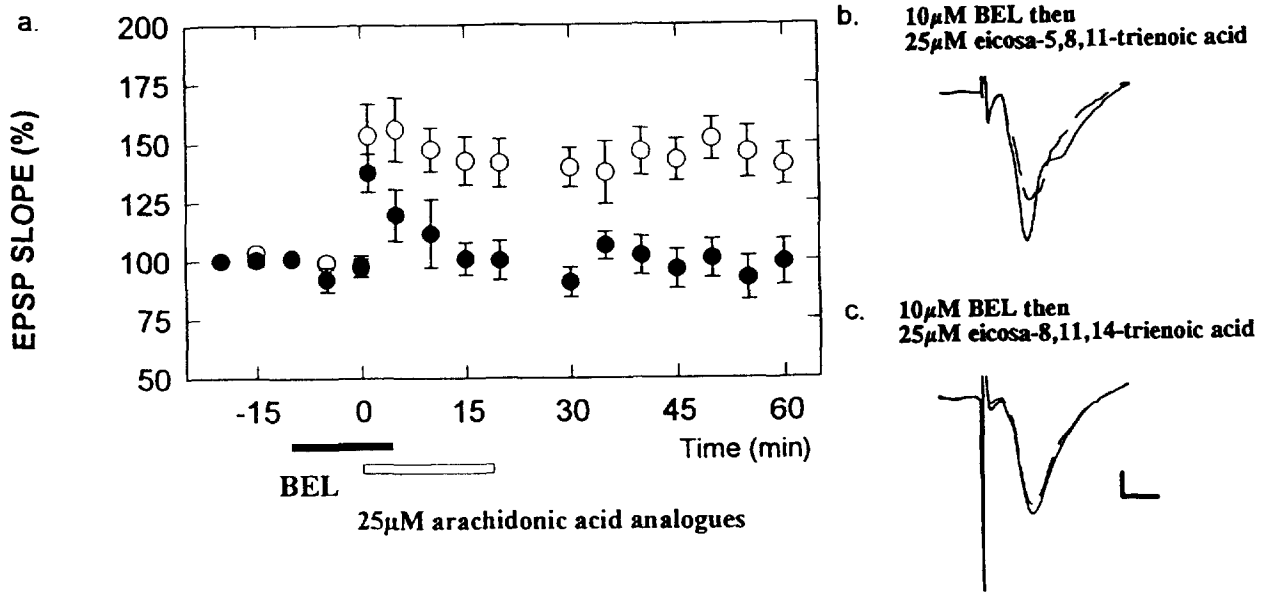

Fig. 4. Eicosa-5,8,11-trienoic acid, but not eicosa-8,11,14-trienoic acid, abolishes the BEL-mediated inhibition of hippocampal LTP. (A) The time course of the change in EPSP slope was determined for slices treated with 10 μM BEL followed by the application of 25 μM eicosa-5,8,11-trienoic acid (open circles) or 25 μM eicosa-8,11,14-trienoic acid (filled circles) immediately after tetanus. The traces depict representative EPSPs before (dashed lines) and 60 min after (solid lines) the tetanus in slices treated with 10 μM BEL before the provision of either eicosa-5,8,11-trienoic acid (B) or eicosa-8,11,14-trienoic acid (C). Calibration bar = 1 mV, 5 ms.

inhibition to identify the importance of calcium-independent phospholipase A_2 in the induction of LTP. Pretreatment of rat hippocampal slices with 10 μM BEL prior to tetanic stimulation resulted in inhibition of the induction of LTP (40.8 ± 5.6% increase in EPSP slope for control (n = 6) vs. 5.8 ± 8.5% increase in EPSP slope for 10 μM BEL (n = 8)) (Fig. 2A). Despite the complete inhibition of EPSP LTP by BEL, 10 μM BEL failed to alter PS LTP in CA1 (50.6 ± 8.5% increase in PS amplitude in control (n = 6) vs. 47.2 ± 16.4% increase in PS amplitude for 10 μM BEL (n = 8)). A previous study similarly found that NDGA, a lipoxygenase inhibitor, blocked EPSP but not PS LTP in the dentate gyrus [25]. In CA1, we also found that 30 μM NDGA blocked EPSP, but not PS, LTP (0.2 ± 3.5% change in EPSP slope and 39.3 ± 13.1% change in PS amplitude, (n = 5)) (data not shown). These results suggest that the release of arachidonic acid by calcium-independent phospholipase A_2 and subsequent oxygenation of the released arachidonic acid through lipoxygenase pathways are crucial for the enhancement of synaptic responses, but not for the potentiation of PS amplitude, following tetanic stimulation.

A detailed examination of the dose-response profile for the inhibition of LTP by BEL demonstrated 50% inhibition of EPSP LTP induction with 1 μM BEL (data not shown). Furthermore, the calcium-dependent phospholipase A_2 inhibitor, $AACOCF_3$ (10 μM) [26,27], as well as a conventionally employed phospholipase A_2 inhibitor, bromophenacyl bromide (50 μM), did not inhibit the induction of LTP (Fig. 2B). A previous study has shown that prolonged application (1 h) of bromophenacyl bromide (50 μM) suppressed LTP induced by theta burst stimulation [28]. However, bromophenacyl bromide is relatively non-specific since it is an activated α-haloketone which has inhibitory effects on multiple enzymes (e.g. [29]). Exposure of hippocampal slices to 10 μM BEL in the presence of CNQX and 0.1 mM Mg^{2+} did not inhibit the NMDA component of EPSPs, thus confirming that BEL did not inhibit LTP by directly affecting NMDA receptor function (Fig. 2C) [30,31].

To substantiate the specificity of BEL mediated inhibition, we demonstrated that the inhibition of LTP by BEL could be rescued by the provision of arachidonic acid, the end product of the calcium-independent phospholipase A_2 reaction. Application of 25 μM arachidonic acid immediately after tetanic stimulation restored the induction of LTP in BEL treated slices to control levels (65.0 ± 28.2% increase in EPSP for 10 μM BEL+25 μM arachidonic acid (n = 5)) (Fig. 3A). Application of 10 μM BEL+25 μM arachidonic acid in the absence of tetanic stimulation had no lasting effect on EPSP or PS amplitude (Fig. 3C) in agreement with prior studies [32].

To further discriminate the selectivity of the inhibition of LTP by BEL, and the importance of other oxidative enzymes potentially involved in this cascade [32–34], we compared the ability of eicosa-5,8,11-trienoic acid and eicosa-8,11,14-trienoic acid to rescue LTP after inhibition by BEL. Remarkably, 25 μM eicosa-5,8,11-trienoic acid, but not 25 μM eicosa-8,11,14-trienoic acid, completely abolished the BEL-mediated inhibition of LTP restoring EPSPs to control levels (−3.7 ± 5.2% increase in EPSP for 10 μM BEL+25 μM eicosa-8,11,14-trienoic acid (n = 4) vs. 37.6 ± 16.1% increase in EPSP for 10 μM BEL+25 μM eicosa-5,8,11-trienoic acid (n = 5)) (Fig. 4A). These results, in conjunction with the ability of NDGA (30 μM) to block EPSP LTP in the CA1 region underscore the importance of calcium-independent phospholipase A_2 mediated arachidonic acid release and its subsequent oxidation (eicosa-5,8,11-trienoic acid but not eicosa-8,11,14-trienoic acid is a substrate for 5′-lipoxygenase [34]) in the induction of hippocampal LTP.

Collectively, these results demonstrate that: (1) calcium-independent phospholipase A_2 is the predominant phospholipase A_2 present in the rat hippocampus; (2) the activation of cal-

cium-independent phospholipase A_2 is necessary, but not sufficient, for the induction of CA1 LTP; and (3) the coupling of calcium-independent phospholipase A_2 mediated-arachidonic acid release to the generation of oxygenated eicosanoid metabolites is critical in executing complex multicellular networked neuronal processes such as LTP and synaptic plasticity.

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