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Arachidonic acid evokes inositol phospholipid hydrolysis in astrocytes

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Astrocyte cultures prelabelled with [^{3}H]inositol were exposed to arachidonic acid (AA) in the presence and absence of various agonists. AA alone evoked a dose-dependent increase in the accumulation of inositol phosphates (IP), an effect not secondary to eicosanoid synthesis and release but which was abolished by EGTA. Separation of the IP revealed that AA stimulated increases in inositol tris-, bis- and monophosphates. IP formation evoked by carbachol or norepinephrine was additive with AA, whereas IP formation by platelet activating factor (PAF) or ATP was non-additive with AA. These results suggest that AA released upon stimulation of astrocytes or other cells in the CNS could initiate and/or amplify intercellular signalling.

Arachidonate; Inositol phosphate; Astrocyte; Phospholipase C; Receptor; Cell signaling

1. INTRODUCTION

Arachidonic acid (AA) is fundamental to a variety of biological functions. AA is a major component of membrane phospholipids, from which it can be liberated directly or indirectly by agonist activation of phospholipases, and serve as a precursor for subsequent eicosanoid synthesis [1]. AA is also implicated in the activation of protein kinase C (PKC), adenylate cyclase, and regulation of intracellular calcium concentration [2]. Support for AA and/or its metabolites as signal molecules has been boosted recently with the finding that K^+ channel opening in cardiac and smooth muscle is so regulated [3]. The proposal that AA itself is a signal molecule is attractive because in many instances agonist stimulation of phospholipases results in the liberation of free AA, in addition to eicosanoids [4]. AA is also implicated in CNS malfunctions. Associated with CNS trauma, there is a marked rise in free fatty acids, particularly in AA, which is considered to be a major mediator of ischemic cell injury [5], possibly in part through the ability of AA to interfere with mitochondrial respiration [6] and the effects of oxygen radicals involved in the metabolism of AA and eicosanoids [7]. As AA itself appears to function as a signal molecule, it is possible that the high levels associated with trauma modify or disrupt normal inter- and intra-cellular signalling processes.

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Prompted by earlier reports [8,9] that AA stimulates phosphoinositide (PPI) hydrolysis by activating phospholipase C (PLC), we have examined the effects of AA on primary cultures of astrocytes, cells which liberate AA upon stimulation [1] and which have been well characterized in terms of receptor-linked PPI turnover [10]. AA directly stimulates the accumulation of inositol phosphates (IP) in these cells, and can amplify the IP response evoked by adrenergic and cholinergic receptor agonists.

2. MATERIALS AND METHODS

Astrocyte-enriched cultures were prepared from newborn rat cerebral cortex [11] and grown to confluence (18 days in vitro) on 6-well plates or in T75 flasks. Immunocytochemical characterization showed that these cultures contained 90–95% glial fibrillary acidic protein-positive cells.

Breakdown of PPI was assessed, in cultures prelabelled with [^{3}H]inositol, by how much intracellular ^{3}H-labelled inositol phosphates (^{3}H-IP) accumulated in the presence of Li^+. Cultures were prelabelled for 18 h in physiological saline solution (PSS) containing 1 μCi/ml myo-[2-^{3}H]inositol (20 Ci/mmol, Amersham). Cultures were washed in PSS, preincubated for 15 min with 5 mM LiCl, and agonists were added for 30 min. Incubations were terminated by removal of the PSS and the addition of ice-cold methanol. The cells were harvested, and the ^{3}H-IP extracted and separated by ion exchange chromatography [11]. Aliquots of the organic phases also were taken to determine the incorporation into lipids, and ^{3}H recovered in IP was standardized to cpm in lipids. In some experiments the individual ^{3}H-IP formed were determined. Incubations were carried out in T75 flasks for 1 min in the absence of Li^+. The labelled glycerophosphoinositol (GPI), inositol monophosphate (IP_1), inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) were separated by ion-exchange chromatography and eluted with formate solutions of increasing strength [11].

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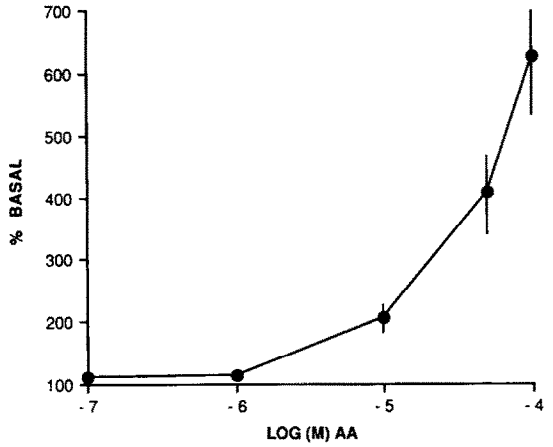

Fig.1. Concentration dependence of AA-stimulated ^{3}H-IP accumulation. Results, expressed as % basal IP accumulation, are means ± SE from 5 experiments.

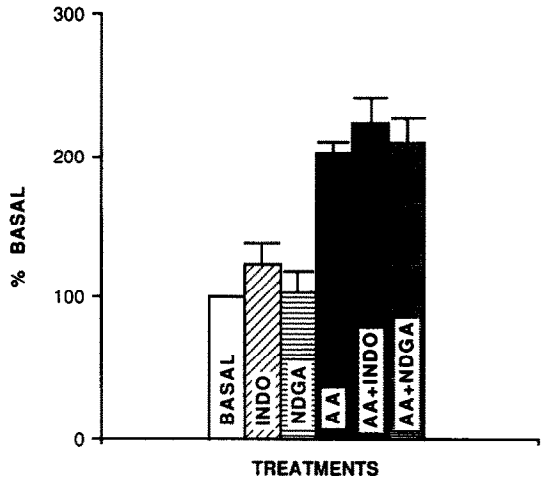

Fig.2. The effects of AA on ^{3}H-IP accumulation in the presence of inhibitors of AA metabolism. AA, 10 μM; INDO, indomethacin, 5 μg/ml; NDGA, nordihydroguaiaretic acid, 5 μM. Results, expressed as % basal IP accumulation, are means ± SE from 3 experiments.

3. RESULTS AND DISCUSSION

Astrocyte cultures incubated with AA (as the free acid) show a concentration-dependent increase in the accumulation of ^{3}H-IP (fig.1), which is two-times the basal value with 10 μM AA rising to six-times the basal value with 100 μM AA. This effect is not due to the vehicle in which the AA is presented to the cells (0.3% ethanol, the maximal concentration achieved, is without effect on IP accumulation), and the effect persists when AA is presented to the cells bound to bovine serum albumin (data not shown). In experiments where cultures were incubated with 10 μM AA in the absence of calcium in the medium and in the presence of 0.5 mM EGTA, IP accumulation was reduced from >200% to 120% of basal. Separation of the individual ^{3}H-IP formed in response to 10 μM AA (n = 3) showed increases (% basal) in IP_3 (143 ± 13), IP_2 (132 ± 5) and IP_1 (147 ± 14), but not GPI (116 ± 20) after 1 min of incubation in the absence of Li^+. These responses were unaffected by EGTA.

An earlier report [8] noted that AA stimulated the phosphatidylinositol phosphodiesterase and so caused the hydrolysis of PPI in a manner which was sensitive to calcium chelators. Zeitler and Handwerger [9] showed that 30 μM AA stimulated a marked increase in IP_1 in placental cells and though they could not detect changes in IP_3, these authors suggested that the time course employed may have missed an initial burst of IP_3 production. Volpi et al. [12], investigating the mechanism of AA-induced calcium mobilization in neutrophils, did not find any PPI hydrolysis with 5 μM AA. Our results are consistent with these data in that AA concentrations of 10 μM and above are required to evoke PPI hydrolysis, and long term IP accumulation is sensitive to calcium chelation. The mechanism by which AA evokes IP accumulation does not seem to require AA entry into the bilayer as AA bound to BSA has similar effects. The possibility of the effect being mediated by AA stimulation of PKC is additionally ruled out as these cells do not show accumulation of IP when exposed to agents (such as phorbol esters) which stimulate PKC directly [13]. The ability of AA to induce IP_3 formation might explain the frequent observation that AA induces intracellular calcium mobilization in numerous cell types, though we have yet to prove causality in astrocytes.

As exogenous AA can drive the synthesis and release of eicosanoids from astrocytes, some of which (thromboxane A_2, prostaglandin F_{2a} and various leukotrienes) evoke the hydrolysis of phosphoinositides [1], we were concerned that the effect of AA on IP accumulation could be secondary to its metabolism. Thus, astrocyte cultures were exposed to AA in the presence of indomethacin and nordihydroguaiaretic acid, inhibitors

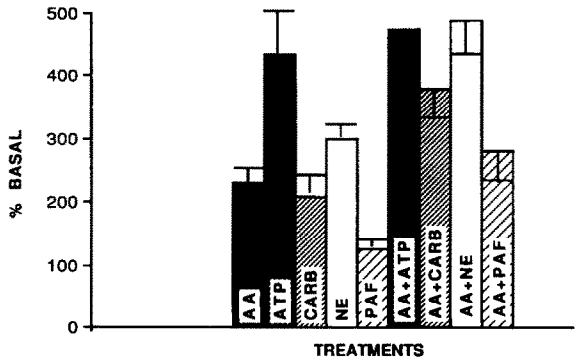

Fig.3. The effects of AA on agonist-evoked ^{3}H-IP formation. AA, 10 μM; ATP, 100 μM; CARB, carbachol, 100 μM; NE, norepinephrine, 100 μM; PAF, platelet activating factor, 100 pM. Results, expressed as % basal IP accumulation, are means ± SE from 3–6 experiments.

of cyclooxygenase and lipoxygenase, respectively. As seen in fig.2, the effect of AA on IP accumulation persisted in the presence of these compounds, suggesting the response is due to a direct action of AA on PPI hydrolysis rather than the result of AA metabolism and eicosanoid release.

In addition to the ability of AA alone to generate IP, we were interested to see the outcome of simultaneous presentation of AA with other PPI-hydrolyzing agonists. A variety of agonists stimulate the turnover of PPI in astrocytes, including norepinephrine (NE) and carbachol [11], ATP [14] and platelet activating factor [15], and we have found that some combinations of agonists are additive, suggesting that receptors are linked to discrete pools of PPI, whereas other combinations are non-additive in their effects on IP accumulation. As seen in fig.3, treatment of astrocytes with AA, NE, carbachol, ATP and PAF stimulated the accumulation of IP with a rank order at these concentrations of ATP $\gg$ NE $>$ carbachol = AA $\gg$ PAF. Combinations of AA with carbachol or NE were additive with respect to IP accumulation, suggesting that separate PPI pools were involved. However, combinations of AA with ATP or PAF were non-additive, the response measured being that of the more effective agonist, and suggesting that AA competes with ATP and PAF for a common pool of PPI.

Many cell types [16–19], including astrocytes [1], release AA together with its metabolites in response to stimulation. This AA could be destined for further processing (to eicosanoids) in neighboring cells [19,20], or it could represent a signal and induce responses in target cells [2]. The observation that AA evokes turnover of PPI and accumulation of IP (including IP_3) in astrocytes, and also amplifies responses in these cells to certain other agonists, is suggestive evidence in favor of the hypothesis that AA itself can function directly as an intercellular signal in the CNS. In conditions such as ischemia or seizure when fatty acid levels are high ($>100\,\mu M$), AA could disrupt normal intercellular signalling and so contribute to CNS dysfunction.

The precise mechanism by which AA stimulates PPI hydrolysis remains to be determined. It is possible that AA interacts with an eicosanoid receptor linked to PPI hydrolysis, suggested by recent findings of Naccache et al. [21] that part of the AA-induced mobilization of calcium in neutrophils is mediated by interaction of the fatty acid with the leukotriene B_4 receptor. Evidence is therefore accumulating to suggest that AA be considered an autacoid in its own right.

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