

PKC-independent inhibition of glutamate exocytosis by arachidonic acid in rat cerebrocortical synaptosomes

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In rat cerebrocortical synaptosomes, the addition of 4 β -phorbol dibutyrate (4 β -PDBu) and arachidonic acid enhances and decreases, respectively, the glutamate release evoked by 4-aminopyridine. Pretreatment of synaptosomes with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or preincubation with staurosporine, prevent the stimulatory effect of 4 β -PDBu, but are without effect on the inhibitory action of arachidonic acid. Moreover, methyl arachidonate, which is not effective as a PKC activator, also strongly inhibits glutamate exocytosis. These results suggest that PKC is not involved in the inhibition of glutamate release by arachidonic acid.

Protein kinase C; Glutamate exocytosis; Arachidonic acid; Synaptosome; Rat brain

1. INTRODUCTION

Protein kinase C (PKC) is a family of enzymes which have been proposed to play an important role in several neuronal functions such as transmitter release, long-term potentiation and the modulation of ionic channels [1,2]. PKC is activated by phorbol esters, such as 12-*O*-tetradecanoylphorbol 13 acetate (TPA), that mimic the physiological activator diacylglycerol [3]. More recently, it has been demonstrated that PKC can also be activated by *cis*-fatty acids [4–6]. It is well known that the activation of PKC with phorbol esters enhances the release of a variety of neurotransmitters [7–11], including glutamate [12–14]. In cerebrocortical synaptosomes, the addition of phorbol esters greatly enhances the release of transmitter glutamate induced by depolarization with 4-aminopyridine [14]. However, we have recently found that arachidonic acid inhibits the 4AP-evoked release of glutamate [15,16]. The present report describes experiments conducted to determine whether the inhibition of glutamate exocytosis by arachidonic acid is mediated by PKC activation. The results indicate a PKC-independent inhibition of glutamate release by arachidonic acid.

Abbreviations: 4AP, 4-aminopyridine; 4 β -PDBu, 4 β -phorbol dibutyrate; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)-amino]ethane sulphonate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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2. MATERIALS AND METHODS

2.1. Preparation of synaptosomes

Synaptosomes were prepared from the cerebral cortices of male Wistar rats [17]. Synaptosomes (1 mg pellets) were resuspended into 1.5 ml of incubation medium (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 1.2 mM MgSO₄, 10 mM glucose and 20 mM TES buffer, pH 7.4) and incubated for 1 h at 37°C in the presence of 16 μ M bovine serum albumin (BSA), essentially fatty acid free (Sigma), to prevent the effects of free fatty acids released from synaptosomes during the incubation [15].

2.2. Glutamate release

Glutamate release was determined as previously described [18,19]. After pre-incubation for 1 h in the presence of BSA, the synaptosomes were pelleted and resuspended in fresh incubation medium without albumin. An aliquot (1 ml) was transferred to a stirred cuvette containing 1 mM NADP, 50 U of glutamate dehydrogenase, and 1.33 mM CaCl₂ or 200 nM free [Ca²⁺] (50 μ M EGTA and 38 μ M CaCl₂ [20]). The fluorescence was measured using a Perkin Elmer model LS-50 luminescence spectrometer.

2.3. TPA preincubation

Synaptosomes were exposed to 1 μ M TPA for 30 min and the incubation stopped by placing the tubes on ice. Samples were washed by centrifugation at 17,000 \times g for 10 min. After pre-incubation for 30 min in the presence of BSA, the synaptosomes were pelleted and resuspended in fresh incubation medium without albumin and assayed for glutamate release.

2.4. Materials

Ficoll was from Pharmacia (Uppsala, Sweden). 4 β -PDBu, TPA, glutamate dehydrogenase, NADP, arachidonic acid and methyl arachidonate were from Sigma (St. Louis, MO, USA). Arachidonic acid was stored under nitrogen atmosphere at -70°C as a 15 mM stock suspension in water and occasionally in dimethyl sulfoxide. Methyl arachidonate was prepared in dimethyl sulfoxide.

3. RESULTS AND DISCUSSION

The addition of low concentrations of arachidonic

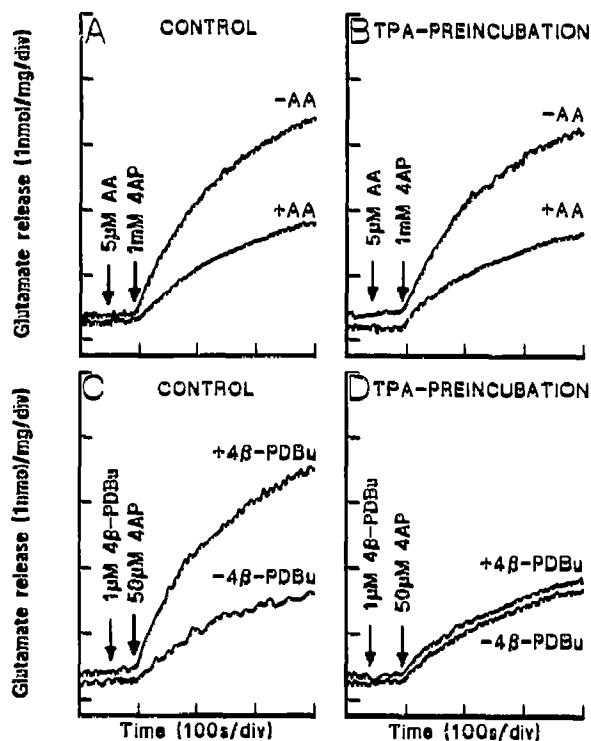


Fig. 1. TPA-pre-incubation prevents the 4β -PDBu-induced enhancement of glutamate release but not the inhibitory action of arachidonic acid. Synaptosomes were exposed to $1\ \mu\text{M}$ PMA for 30 min as detailed in Materials and Methods. Control synaptosomes were treated identically in the absence of TPA. Glutamate release in control (A and C) and TPA-pre-incubated synaptosomes (B and D) was measured in the presence of $1.33\ \text{mM}$ CaCl_2 .

acid to rat cerebrocortical synaptosomes strongly inhibited the release of glutamate evoked by depolarization with a maximal concentration of 4-aminopyridine (Fig. 1A), while the addition of the phorbol ester, 4β -PDBu, greatly enhanced the release of transmitter glutamate evoked by a submaximal concentration of 4AP (Fig. 1C). In order to know whether inhibition of glutamate exocytosis by arachidonic acid was mediated by PKC activation we pre-incubated the synaptosomes with $1\ \mu\text{M}$ TPA for 30 min. The treatment of synaptosomes with high concentrations of TPA has been shown to down-regulate the PKC activity [11,13]. After 30 min exposure to $1\ \mu\text{M}$ TPA followed by washing and resuspension, synaptosomes responded normally in terms of glutamate release to $50\ \mu\text{M}$ 4AP but did not respond to $1\ \mu\text{M}$ 4β -PDBu added 1 min prior to 4AP (Fig. 1D). In agreement with these results it has been shown that pretreatment of synaptosomes with TPA does not alter the K^+ -evoked noradrenaline release, but the ability of TPA to enhance the evoked release is lost [11]. In contrast to 4β -PDBu experiments, the ability of arachidonic acid to reduce glutamate exocytosis was not altered by pretreatment with TPA (Fig. 1B).

Cerebral cortical synaptosomes contain the α , β and γ subspecies of PKC [6]. After the treatment of synaptosomes from cerebrum with TPA, the rate of depletion

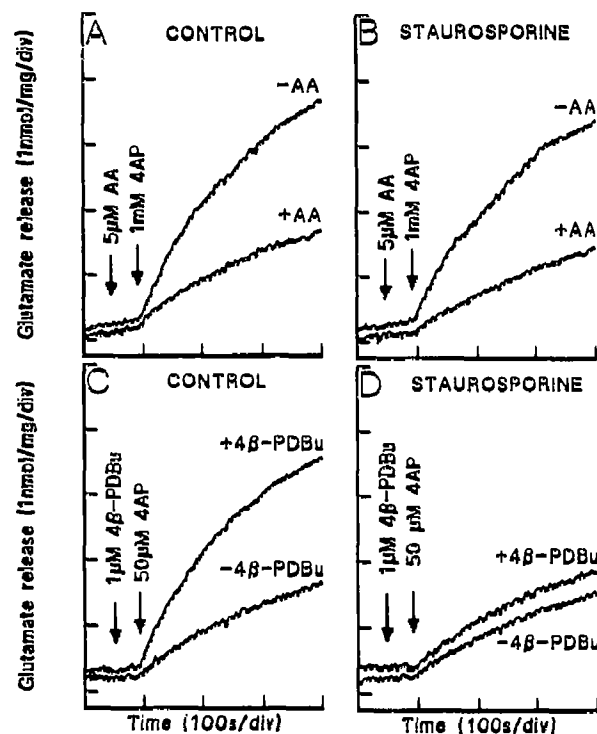


Fig. 2. Staurosporine prevents the 4β -PDBu-induced enhancement of glutamate release but not the inhibitory action of arachidonic acid. Synaptosomes were pre-incubated in the absence (A and C) and presence (B and D) of $1\ \mu\text{M}$ staurosporine for 30 min and the glutamate release measured in the presence of $1.33\ \text{mM}$ CaCl_2 .

of α and β subspecies has been shown to be very rapid, while some activity of the γ subspecies is retained [11]. It could be argued, therefore, that the remaining activity of PKC in TPA-treated synaptosomes might be responsible for the inhibition of arachidonic acid on glutamate release. An alternative strategy in establishing whether a given effect is mediated by PKC is to use protein kinase inhibitors. Unfortunately, none of the inhibitors is completely specific for PKC, as they all inhibit Ca^{2+} -calmodulin protein kinase or cyclic nucleotide kinase to

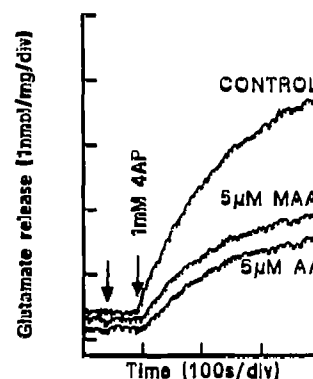


Fig. 3. Methyl arachidonate also inhibits glutamate release. Glutamate release was estimated in the presence of $1.33\ \text{mM}$ CaCl_2 . When indicated, arachidonic acid (AA) and methyl arachidonate (MAA) were added 1 min prior to depolarization with $1\ \text{mM}$ 4AP.

some degree [21,22]. Keeping this in mind, Fig. 2 shows that staurosporine prevents the phorbol esters-induced enhancement of glutamate release but, again, the inhibitory action by arachidonic acid is present. It is well established that protein kinase C activation is specific to the *cis*-form of free fatty acids [6]. However, glutamate exocytosis was strongly inhibited not only by arachidonic acid, but also by methyl arachidonate (Fig. 3), which has been shown not to be effective as a protein kinase C activator [4]. In our experiments the ability of methyl arachidonate to inhibit glutamate exocytosis was not due to the disruption of synaptic plasma membranes, since methyl arachidonate did not modify the Ca^{2+} -independent release of cytoplasmic glutamate (data not shown).

We have recently reported that the presynaptic inhibition by arachidonic acid of glutamate exocytosis seems to be mediated by the modulation of K^{+} -channels that control the duration of the action potentials induced by 4-aminopyridine, causing a reduced depolarization and Ca^{2+} -entry, and thereby resulting in a reduced transmitter release [16]. In the present study, we present data suggesting that the inhibitory effect by fatty acids in glutamate release is independent of PKC-activation. An alternative explanation for the inhibition of glutamate release in cerebrocortical synaptosomes is by a direct action of arachidonic acid or its oxygenated metabolites on presynaptic K^{+} -channels, as has been demonstrated for a number of channel types in a variety of preparations [23–26]. However, the demonstration of such a mechanism in cortical nerve terminals remains to be established.

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