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

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In rat cerebrocortical synaptosomes, the addition of 4β-phorbol dibutyrate (4β-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 pre-incubation with staurosporine, prevent the stimulatory effect of 4β-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 PCK activation. The results indicate a PCK-independent inhibition of glutamate release by arachidonic acid.

Abbreviations: 4AP, 4-aminopyridine; 4β-PDBu, 4β-phorbol dibutyrate; $[Ca^{2+}]$, cytosolic free Ca^{2+} 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 KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose and 20 mM TES buffer, pH 7.4) and incubated for 1 h at 37°C in the presence of 16 μM 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 $CaCl_2$ or 200 nM free $[Ca^{2+}]$ (50 μM EGTA and 38 μM $CaCl_2$ [20]). The fluorescence was measured using a Perkin Elmer model LS-50 luminescence spectrometer.

2.3. *TPA preincubation*

Synaptosomes were exposed to 1 μM TPA for 30 min and the incubation stopped by placing the tubes on ice. Samples were washed by centrifugation at 17,000 × *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). 4β-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

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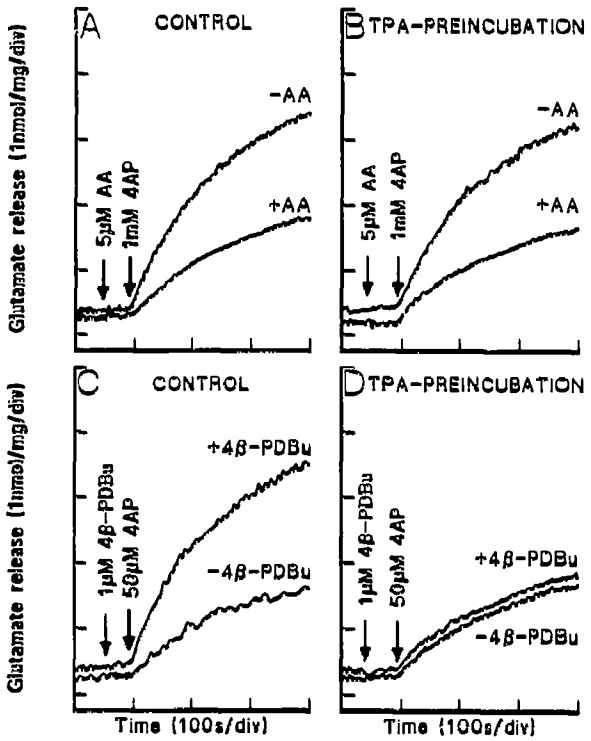

Fig. 1. TPA-pre-incubation prevents the 4β-PDBu-induced enhancement of glutamate release but not the inhibitory action of arachidonic acid. Synaptosomes were exposed to 1 μM PMA for 30 min as detailed in Materials and Methods. Control synaptosomes were treated identically in the absence of TPA. Glutamate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

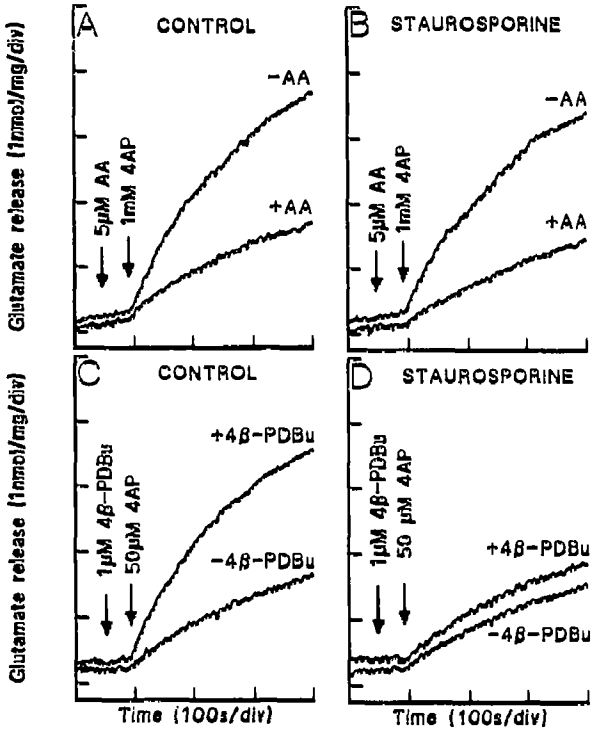

Fig. 2. Staurosporine prevents the 4β-PDBu-induced enhancement of glutamate release but not the inhibitory action of arachidonic acid. Synaptosomes were pre-incubated in the absence (A and C) and presence (B and D) of 1 μM staurosporine for 30 min and the glutamate release measured in the presence of 1.33 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 μM 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 μM TPA followed by washing and resuspension, synaptosomes responded normally in terms of glutamate release to 50 μM 4AP but did not respond to 1 μM 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 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

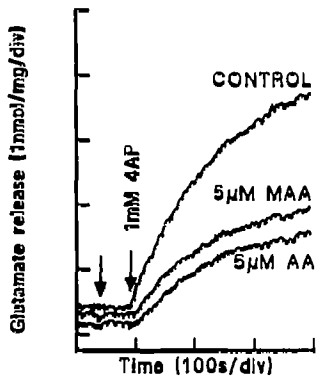

Fig. 3. Methyl arachidonate also inhibits glutamate release. Glutamate release was estimated in the presence of 1.33 mM $CaCl_2$. When indicated, arachidonic acid (AA) and methyl arachidonate (MAA) were added 1 min prior to depolarization with 1 mM 4AP.

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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REFERENCES

[1] Nishizuka, Y. (1986) Science 233, 305–312.
[2] Nishizuka, Y. (1988) Nature 334, 661–665.
[3] Castagna, M., Takai, Y., Kakuichi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[4] Murakami, K. and Routtenberg, A. (1985) FEBS Lett. 192, 189–193.
[5] Murakami, K., Chan, S.Y. and Routtenberg, A. (1986) J. Biol. Chem. 261, 15424–15429.
[6] Shearman, M.S., Shinomura, T., Oda, T. and Nishizuka, Y. (1991) J. Neurochem. 56, 1255–1262.
[7] Zurgil, N. and Zisapel, N. (1986) FEBS Lett. 65, 257–261.
[8] Nichols, R.A., Haycock, J.W., Wang, J.K.T. and Greengard, P. (1987) J. Neurochem. 48, 615–621.
[9] Shu, C. and Selmanoff, M. (1988) Endocrinology 122, 2699–2709.
[10] Shuntoh, H., Taniyama, K., Fukuzaki, H. and Tanaka, C. (1988) J. Neurochem. 51, 1565–1572.
[11] Oda, T., Shearman, M.S. and Nishizuka, Y. (1991) J. Neurochem. 56, 1263–1269.
[12] Lynch, M.A. and Bliss, T.V.P. (1986) Neurosci. Lett. 65, 171–176.
[13] Díaz-Guerra, M.J.M., Sánchez-Prieto, J., Boscá, L., Pocock, J., Barrie, A. and Nicholls, D. (1988) Biochim. Biophys. Acta 970, 157–165.
[14] Barrie, A.P., Nicholls, D.G., Sánchez-Prieto, J. and Sihra, T.S. (1991) J. Neurochem. 57, 1394–1404.
[15] Herrero, I., Castro, E., Miras-Portugal, M.T. and Sánchez-Prieto, J. (1991) Neurosci. Lett. 126, 41–44.
[16] Herrero, I., Miras-Portugal, M.T. and Sánchez-Prieto, J. (1991) J. Neurochem. 57, 718–721.
[17] Nicholls, D.G. (1978) Biochem. J. 170, 511–522.
[18] Nicholls, D.G., Sihra, T.S. and Sánchez-Prieto, J. (1987) J. Neurochem. 49, 50–57.
[19] Rubio, I., Torres, M., Miras-Portugal, M.T. and Sánchez-Prieto, J. (1991) J. Neurochem. 59, 1159–1164.
[20] Verhage, M., Besselsen, E., Lopes da Silva, F.H. and Ghijsen, W.E.J.M. (1989) J. Neurochem. 53, 1188–1194.
[21] Linden, D.J. and Routtenberg, A. (1989) J. Physiol. 419, 95–119.
[22] Yanagihara, N., Tachikawa, E., Izumi, F., Yasugawa, S., Yamamoto, H. and Miyamoto, E.J. (1991) J. Neurochem. 56, 294–298.
[23] Piomelli, D., Volterra, A., Dale, N., Seigelbaum, S.A., Kandel, E.R., Schwartz, J.H. and Belardetti, F. (1987) Nature 328, 38–43.
[24] Kim, D., Lewis, D.L., Graziadei, L., Neer, E.J., Bar-Sagi, D. and Clapham, D.E. (1989) Nature 557–560.
[25] Ordway, R.W., Walsh Jr., J.V. and Singer, J.J. (1989) Science 244, 1176–1179.
[26] Ordway, R.W., Singer, J.J. and Walsh Jr., J.V. (1991) Trends Neurosci. 14, 96–100.