

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Avenue, Washington, DC 20503.

1. REPORT DATE
1990

3. REPORT TYPE AND DATES COVERED
Reprint

AD-A227 662

5. FUNDING NUMBERS
Program Element No.
NWED QAXM

6. AUTHOR(S)

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Work Unit No.
00157

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Armed Forces Radiobiology Research Institute
Defense Nuclear Agency
Bethesda, MD 20889-5145

8. PERFORMING ORGANIZATION
REPORT NUMBER

SR90-12

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Defense Nuclear Agency
Washington, DC 20305

10. SPONSORING/MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

DTIC
ELECTE
OCT 12 1990
S E D

14. SUBJECT TERMS

15. NUMBER OF PAGES

5

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

UNCLASSIFIED

18. SECURITY CLASSIFICATION
OF THIS PAGE

UNCLASSIFIED

19. SECURITY CLASSIFICATION
OF ABSTRACT

20. LIMITATION OF
ABSTRACT

DTIC FILE COPY

SECURITY CLASSIFICATION OF THIS PAGE

CLASSIFIED BY

DECLASSIFY ON:

SECURITY CLASSIFICATION OF THIS PAGE

ARACHIDONIC ACID AND PROSTAGLANDINS ENHANCE POTASSIUM-STIMULATED CALCIUM INFLUX INTO RAT BRAIN SYNAPTOSOMES

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(Accepted 2 March 1990)

Summary—Exogenous administration of arachidonic acid, prostaglandins $\text{PGF}_2\alpha$, PGD_2 and PGE_2 increased potassium-stimulated uptake of calcium in rat brain synaptosomes from the brain of the rat, but had no effect on the basal uptake of calcium. Arachidonic acid-induced uptake of calcium was mediated by its prostaglandin metabolites, because a cyclooxygenase inhibitor, indomethacin, inhibited the response. L-Type calcium channel blockers, such as verapamil, diltiazem and nimodipine, blocked both KCl - and prostaglandin-enhanced potassium-stimulated influx of calcium in the brain. These results suggest that prostaglandins act as a calcium ionophore, through L-type voltage-sensitive calcium channels.

Key words—arachidonic acid, brain, calcium, prostaglandins, synaptosomes.

The formation of prostaglandins, prostacyclin, thromboxanes or leukotrienes in different cells require arachidonic acid, a naturally occurring *cis*-polyunsaturated fatty acid that is found primarily esterified to membrane lipids in mammalian cells (Samuelson, 1981). The release of arachidonic acid from lipids may be an early step in the mechanism of action of some secretagogues (Samuelson, 1981). Prostaglandins are synthesized in response to various stimuli and are known to modulate numerous physiological functions, including central ones (Chiu and Richardson, 1985; Oliv, Granstrom and Anggard, 1983). Prostaglandins have been demonstrated to be involved in thermoregulation (Milton and Wendlandt, 1970; Ueno, Narumiya, Ogorochi, Nakayama, Ishikawa and Hayaishi, 1982), induction of sleep (Ueno, Ishikawa, Nakayama and Hayaishi, 1982; Ueno, Honda, Inoue and Hayaishi, 1983; Ueno, Osama, Urade and Hayaishi, 1985), nociception (Horiguchi, Ueno, Hyodo and Hayaishi, 1986; Ohkubo, Shibata, Takahashi and Inoki, 1983), anticonvulsive effects (Forestman, Heldt, Knappen and Hertting, 1982), release of neurotransmitters (Higashida, Nakagawa and Miki, 1984; McGee, Simpson, Christain, Mata, Nelson and Nirenberg, 1978) and other actions (Wolfe, 1982; Wolfe and Coccani, 1979). In addition, prostaglandins elevate levels of 3',5'-cyclic adenosine monophosphate cyclic (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) in neural tissue (Gilman and Nirenberg, 1971; Hamprecht and Schultz, 1973).

Calcium is important in a variety of functions, in both the central nervous system and in peripheral tissues. In neural tissue, the influx of calcium into presynaptic nerve endings initiates events leading to

release of neurotransmitter and in heart and in smooth muscle, calcium couples excitation to contraction. The entry of calcium into these tissues appears to be mediated by voltage-sensitive calcium channels (Hagiwara and Byerly, 1981). Several classes of compounds have been shown to interact with these channels. The most important class has been the dihydropyridine calcium antagonists, such as nitrendipine and nimodipine. Drugs from other chemical classes, such as verapamil and diltiazem, also act as calcium antagonists (Schwartz, 1982).

Because arachidonic acid and/or its metabolites may be intracellular effectors of calcium-mediated secretion, the effect of exogenous administration of arachidonic acid and prostaglandins on basal and potassium-stimulated influx of calcium into synaptosomes was studied. In addition, the effect of calcium channel blockers on the uptake of calcium was studied.

METHODS

Materials

Arachidonic acid, prostaglandins $\text{PGF}_2\alpha$, PGE_2 and PGD_2 , verapamil hydrochloride, diltiazem hydrochloride and indomethacin were purchased from Sigma (St Louis, Missouri) and were dissolved in ethanol. Nimodipine was a gift from Miles Laboratories Inc. (New Haven, Connecticut) and was dissolved in dimethyl sulphoxide (DMSO). Because nimodipine is sensitive to light, experiments were done using amber-colored test tubes. The $^{45}\text{CaCl}_2$ was purchased from New England Nuclear.

Preparation of tissue and uptake of calcium

Male Sprague Dawley rats, weighing 200–300 g (Charles River Breeding Laboratories, Kingston, New York), were used in these experiments. The crude synaptosomal (P_2) fraction was prepared by a modification of the method of Gray and Whittaker, 1962. The final pellet was resuspended in ice-cold incubation medium (NaCl, 136 mM; KCl, 5 mM; CaCl_2 , 0.12 mM; MgCl_2 , 1.3 mM; glucose, 10 mM; Tris base 20 mM; pH adjusted to 7.65 with 1.0 M maleic acid), to provide a concentration range of approximately 4–6 mg protein/ml.

For the determination of the uptake of calcium, 0.48 ml of the synaptosomal preparation was pipetted into test tubes and incubated for approximately 14 min at 30 °C in a Dubnoff metabolic shaker, in the presence or absence of various concentrations of drugs, as described later. The drugs were added in a 20- μ l volume to make final incubation volume of 0.5 ml. For control samples, 20 μ l of incubation medium, with various concentrations of ethanol or DMSO, were added. For these experiments, 0.5 ml of depolarizing or nondepolarizing solution, containing $^{45}\text{Ca}^{2+}$ (3 μCi) was added for 3 sec. The uptake of calcium was terminated by the prompt addition of 5 ml of an ice-cold EGTA stopping solution (NaCl, 136 mM; KCl, 5 mM; MgCl_2 , 1.3 mM; EGTA [ethylene-glycol-bis-(beta-amino-ethyl) ether] N,N' -tetraacetic acid], 3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1.0 M maleic acid). The composition of the depolarizing solution was the same as that of the incubation medium, except that a portion of the NaCl was isosmotically replaced by KCl, to provide a final concentration of KCl of 65 mM. The nondepolarizing solution had the same composition as the incubation medium, except for the presence of $^{45}\text{Ca}^{2+}$. Depolarizing and nondepolarizing solutions, added to drug-exposed synaptosomes, also contained the same concentrations of drug, to maintain the designated molar relationships. Each sample was immediately filtered under vacuum through a Whatman GF/B filter, presoaked with nondepolarizing solution. Each filter was then washed with two 5-ml aliquots of ice-cold incubation medium and placed in a scintillation vial. Radioactivity was determined by liquid scintillation spectrometry.

To calculate the net uptake of $^{45}\text{Ca}^{2+}$ into synaptosomes, the uptake in the absence of depolarization (5 mM KCl) was subtracted from the uptake in the presence of depolarization (65 mM KCl). This value is referred to as 'k' (potassium-induced change) and represents net KCl-induced uptake of calcium (Blaustein and Fodor, 1975; Leshe, Friedman, Wilcox and Elrod, 1980). Each control and drug experiment was performed using matched synaptosomal preparations from the same batch. The content of protein was determined by the method of Lowry, Rosebrough, Farr and Blaustein, 1951), using bovine

serum albumin as the standard. All data are expressed as the mean $\pm$ SEM of triplicate determinations. Values for the effect of indomethacin or calcium channel antagonists on the enhancement by arachidonic acid, $\text{PGF}_2\alpha$, PGD_2 or PGE_2 of the potassium-stimulated uptake of calcium were represented as a percentage of control, i.e. responses to arachidonic acid, $\text{PGF}_2\alpha$, PGD_2 or PGE_2 in the absence of indomethacin or calcium channel antagonists. Statistical analyses were performed using analysis of variance (ANOVA) (RS1; BBN Software Products Corp., Cambridge, MA), for identifying the main effects, along with subsequent *post hoc* tests, when appropriate. Data were identified as significant if $P < 0.05$.

RESULTS

Prostaglandin $\text{PGF}_2\alpha$ (10 nM–10 μM) and arachidonic acid, PGD_2 and PGE_2 (1–30 μM each) increased the entry of potassium-stimulated influx of calcium (Fig. 1) but had no effect on the basal uptake of calcium (data not shown). Arachidonic acid (2 μM)-induced uptake of calcium was inhibited by indomethacin (1–10 μM) (Fig. 2), a cyclooxygenase inhibitor, suggesting that arachidonic acid acted through the formation of prostaglandins. However, indomethacin had no inhibitory effect on the enhancement by $\text{PGF}_2\alpha$, PGE_2 and PGD_2 of the KCl-stimulated influx of calcium (data not shown). Preincubation of the synaptosomes with the voltage-sensitive channel blockers verapamil (5–30 μM), nimodipine (1–10 μM) or diltiazem (50–300 μM), inhibited the potassium- $\text{PGF}_2\alpha$ - or PGD_2 - and PGE_2 -enhanced potassium-stimulated influx of calcium (Table 1).

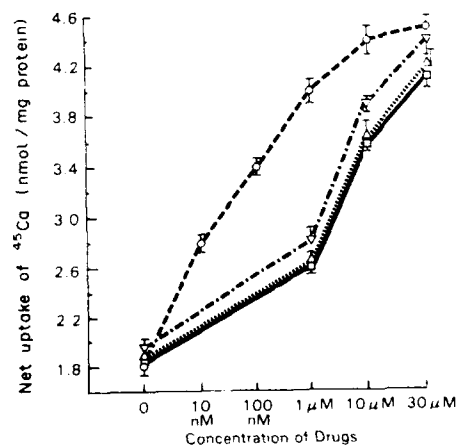


Fig. 1. Effect of arachidonic acid (□), $\text{PGF}_2\alpha$ (○), PGD_2 (▽) or PGE_2 (△) on potassium-stimulated uptake of calcium in synaptosomes from the brain of the rat. Points and bars represent mean $\pm$ SEM values from three separate experiments, each using triplicate samples.

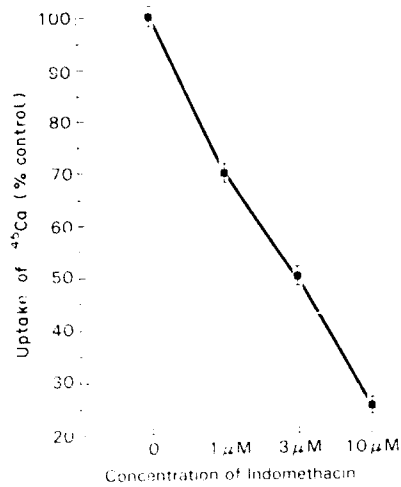


Fig. 2 Effect of indomethacin (■) on arachidonic acid (3 μ M)-induced potassium-stimulated uptake of calcium in synaptosomes from the brain of the rat. Values are mean $\pm$ SEM of 3 separate experiments, each using triplicate samples, represented as a percentage of control response, in the absence of indomethacin. Net uptake of calcium evoked by 3 μ M arachidonic acid was 2.8 ± 0.05 nmol/mg protein.

DISCUSSION

Synaptosomes provide a useful system for the study of the biochemical mechanisms that mediate stimulation-secretion coupling in neurons. The uptake of $^{45}\text{Ca}^{2+}$ into synaptosomes was comparable on an nmol/mg basis to other reports (Leslie, McCormick and Gonzales, 1982; Leslie, Barr and Chandler, 1983). Potassium-induced depolarization of synaptosomes from the brain of the rat stimulates endogenous phospholipase A₂, which, in turn, releases arachidonic acid from the phospholipids (Bradford,

Marinetti and Abood, 1983). The released arachidonic acid may then be metabolized by lipoxygenase or cyclooxygenase to form leukotrienes, hydroxy-eicosatetraenoic acids or prostaglandins (Samuelson, 1981).

It is possible that some metabolites of arachidonic acid (lipoxygenase products) act as intracellular second messengers for presynaptic inhibition, while others (cyclooxygenase products) act as first messengers on the postsynaptic cell for stimulation (Piomelli, Shapiro, Feinmark and Schwartz, 1987a).

Pharmacological experiments, performed on the identified *Aplysia* interneuron L10 (Shapiro, Piomelli and Schwartz, 1987) and on *Aplysia* sensory cells (Piomelli *et al.*, 1987a; Piomelli, Volterra, Dale, Siegelbaum, Kandel, Schwartz and Belardetti, 1987b), have demonstrated the involvement of lipoxygenase products of arachidonic acid in presynaptic inhibition. In both types of cells, the responses to inhibitory transmitters were mimicked when arachidonic acid was applied (Piomelli *et al.*, 1987a). Likewise, cyclooxygenase products of arachidonic acid are first messengers on the postsynaptic cell for stimulation (Piomelli *et al.*, 1987a) and have been implicated as the biologically active factor(s), because cyclooxygenase inhibitors were shown to inhibit stimulated secretion, while the exogenous administration of one of several metabolites of cyclooxygenase was shown to reproduce the biological response (Whitting and Barritt, 1982). However, many have provided evidence in support of the idea that arachidonic acid itself may be biologically active (Kolesnick, Musacchio, Thaw and Gershengorn, 1984).

Because arachidonic acid and its metabolites have been shown to affect calcium homeostasis in several types of cell (Barritt, 1981), it is possible that secretion, stimulated by arachidonic acid or its

Table 1. Effect of calcium antagonists on the enhancement by $\text{PGF}_2\alpha$, PGD_2 or PGE_2 of the potassium chloride-stimulated uptake of calcium or potassium chloride-stimulated uptake of calcium in synaptosomes from the brain of the rat

Concentration of calcium antagonists	Concentration of prostaglandins and KCl			
	100 nM $\text{PGF}_2\alpha$	3 μ M PGD_2	3 μ M PGE_2	65 mM KCl
Verapamil				
5 μ g	$85\% \pm 0.03$	$90\% \pm 0.07$	$88\% \pm 0.01$	$78\% \pm 0.02^*$
10 μ g	$65\% \pm 0.01^*$	$68\% \pm 0.02^*$	$70\% \pm 0.01^*$	$50\% \pm 0.02^*$
50 μ g	$50\% \pm 0.01^*$	$45\% \pm 0.02^*$	$50\% \pm 0.01^*$	$30\% \pm 0.02^*$
Nifedipine				
1 μ M	$80\% \pm 0.01^*$	$82\% \pm 0.02^*$	$80\% \pm 0.03^*$	$78\% \pm 0.02^*$
3 μ M	$68\% \pm 0.02^*$	$68\% \pm 0.01^*$	$66\% \pm 0.01^*$	$52\% \pm 0.02^*$
10 μ M	$40\% \pm 0.01^*$	$40\% \pm 0.01^*$	$35\% \pm 0.02^*$	$25\% \pm 0.01^*$
Diltiazem				
50 μ M	$85\% \pm 0.01$	$90\% \pm 0.02$	$88\% \pm 0.02$	$72\% \pm 0.02^*$
100 μ M	$65\% \pm 0.02^*$	$68\% \pm 0.02^*$	$67\% \pm 0.01^*$	$50\% \pm 0.02^*$
300 μ M	$48\% \pm 0.02^*$	$47\% \pm 0.02^*$	$50\% \pm 0.02^*$	$35\% \pm 0.02^*$

Values are mean $\pm$ SEM of three separate experiments, each using triplicate samples, represented as a percentage of control ($\text{PGF}_2\alpha$, PGD_2 , PGE_2 or KCl) response in the absence of calcium antagonists. Net uptake of calcium by 100 nM $\text{PGF}_2\alpha$ was 2.7 ± 0.03 nmol/mg, 3 μ g PGD_2 was 2.7 ± 0.05 nmol/mg, 3 μ M PGE_2 was 2.6 ± 0.03 nmol/mg and 65 mM KCl was 1.9 ± 0.03 nmol/mg protein.

*Significantly different from control ($\text{PGF}_2\alpha$, PGD_2 , PGE_2 or KCl) value, without calcium antagonists. $P < 0.05$.

metabolites, is mediated by calcium. Inhibition of arachidonic acid-induced influx of calcium by indomethacin in this study demonstrated that arachidonic acid acts through its cyclooxygenase metabolites, prostaglandins, in stimulating the influx of calcium, supporting the results of most previous studies. The failure of indomethacin to inhibit prostaglandin-induced influx of calcium suggests that it interferes only with the synthesis of prostaglandins.

The prostaglandins $\text{PGF}_2\alpha$, PGE_2 and PGD_2 are the major ones produced (Abdel-Halim, Hamberg, Sjoquist and Anggard, 1977; Miwa, Sugino, Ueno and Hayaishi, 1988; Narumiya, Ogorochi, Nakao and Hayaishi, 1982; Sun, Chapman and McGuire, 1977) in the CNS and their possible functions in the CNS have been reviewed (Wolfe, 1982; Wolfe and Coceani, 1979). However, it is not known whether prostaglandins in the brain are involved in intracellular calcium homeostasis. In this study, $\text{PGF}_2\alpha$, PGD_2 and PGE_2 did not enhance the basal influx of calcium, which contradicts the results in neuroblastoma of the mouse and glioma hybrid NG108-15 cells in the rat, where prostaglandins also induce the influx of calcium (Miwa *et al.*, 1988). However, in the present study, these prostaglandins only enhanced the potassium-stimulated influx of calcium indicating that prostaglandins cannot act directly to promote the influx of calcium. At the present time, it cannot be explained why prostaglandins have no effect on the basal levels of uptake of calcium; however, some suggest that it is due to the inhibition evoked by the released arachidonic acid, by depolarization (Lazarewicz, Leu, Sun and Sun, 1983; Piomelli *et al.*, 1987a).

Although the calcium channel antagonists nimodipine, verapamil and diltiazem represent a chemically heterogeneous group of agents (Schwartz, 1982), they act through a common locus: the voltage-sensitive calcium channel. Several types of calcium channels exist and can be distinguish both physiologically and pharmacologically. The calcium channel blockers exert their actions at the L-class of channel, which is characterized by large conductances, of long duration. Radioligand binding and chemical studies have demonstrated that nimodipine, verapamil and diltiazem interact at discrete sites, associated with a major protein of the calcium channel (Triggle and Janis, 1984; Triggle and Janis, 1987). This protein, the α_1 subunit, with a molecular weight of 170–195 kdaltons, is one of several components of the L-type calcium channel (Catterall, 1988).

Calcium channel blockers, used in this study, blocked the potassium-stimulated influx of calcium and the prostaglandin-enhanced, potassium-stimulated influx of calcium, suggesting that, in the brain, prostaglandins and potassium may activate L-type voltage-sensitive calcium channels. These results support the finding that nifedipine, another dihydropyridine calcium channel blocker, suppressed the uptake of calcium induced by $\text{PGF}_2\alpha$ (Koyama,

Kitayama, Dohi and Tsujimoto, 1988) in a primary culture of bovine adrenal chromaffin cells.

In summary, these results indicate that arachidonic acid induces the uptake of calcium in the brain, an effect that is mediated through the formation of prostaglandins and that the potassium-stimulated influx of calcium and prostaglandin-enhanced, potassium-stimulated influx of calcium may be mediated through the L-type voltage-sensitive calcium channels.

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Availability Codes	
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