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EFFLUX OF γ-AMINOBUTYRIC ACID FROM AND APPEARANCE OF FREE ARACHIDONIC ACID INSIDE SYNAPTOSOMES

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When synaptosomes were depolarized in the presence of Ca^{2+}, or when Ca^{2+} was added to synaptosomes pretreated with Ca^{2+} ionophore (A23187), free arachidonic acid was clearly increased within synaptosomes, and at the same time an efflux of γ-aminobutyric acid from synaptosomes was observed. Moreover, when synaptosomes labelled with [^{14}C]arachidonic acid were depolarized in the presence of Ca^{2+}, there was a significant decrease in the radioactivity of the fatty acid of phosphatidylinositol and phosphatidylcholine. Exogenously added arachidonic acid, but not other fatty acids, stimulated the efflux of γ-aminobutyric acid in the absence of Ca^{2+}. These observations suggest that the release of arachidonic acid from phospholipids is an intrinsic part of the biochemical mechanism that modulates the γ-aminobutyric acid efflux.

Introduction

The efflux of neurotransmitters from the nerve terminals by the action potential is a key event in synaptic communication; neuronal depolarization activates an influx of Ca^{2+} into the nerve terminals, and the increase in intracellular Ca^{2+} triggers the efflux of a neurotransmitter [1]. Although the molecular mechanism that couples the Ca^{2+} influx to the efflux of neurotransmitter is obscure, it is known that several stimulus-secretion mechanisms are coupled with a specific change in turnover of membrane phospholipids [2–5]. For example, platelets (a stimulus-secretion system) have a mechanism for releasing arachidonic acid from membrane phospholipids, and the released arachidonic acid is rapidly transformed into several oxygenated products now considered to play an important role in platelet aggregation and serotonin efflux [6–7]; these products include various prostaglandins.

In previous experiments with the nerve terminal fraction (synaptosomes), we demonstrated that Ca^{2+} influx induced by depolarization was essential to efflux of γ-aminobutyric acid from synaptosomes [8]. We will discuss here whether synaptosomes have a mechanism for release of arachidonic acid from synaptosomal phospholipids which is triggered by the Ca^{2+} influx, and whether the released arachidonic acid is associated with the γ-aminobutyric acid efflux from synaptosomes.

Materials and Methods

Preparation of synaptosomes. Adult male mice weighing about 20 g were used throughout this work. They were decapitated and the brains were quickly removed, weighed and rinsed with cold 0.9% NaCl. A 10% homogenate in 0.32 M sucrose was prepared in a Teflon-glass homogenizer. Crude mitochondrial fraction containing synaptosomes was prepared by the method of Gray and Whittaker [9]. Synaptosomal fraction was separated

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from the crude mitochondrial fraction on a Ficoll-sucrose gradient according to Sellström et al. [10]. Synaptosomes were pelleted at 90000 × *g* for 20 min.

Ca^{2+} ionophore A23187 treatment of synaptosomes. The ionophore was dissolved in a Ca^{2+}-free Ringer's solution to give a concentration of 10 μM, and synaptosomes were suspended in this solution to give a protein concentration of 5 mg/ml and incubated at 37 °C for 10 min under a stream of 95% O_2/5% CO_2: the Ca^{2+}-free Ringer's solution consisted of NaCl 128 (mM), KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, and glucose 10. The synaptosomes thus treated with the ionophore were pelleted at 23000 × *g* for 20 min.

Determination of free arachidonic acid and of esterified arachidonic acid in lipids. Free arachidonic acid in synaptosome was determined as follows [11]. The aliquots of synaptosome suspension were transferred to centrifuge tubes, and after tricosanoic acid was added as an internal standard, free fatty acids were isolated by three extractions with 5 vols. of diethyl ether. The extracts were combined and evaporated to dryness under nitrogen. The residue was treated with *N,N*-dimethylformamide di-*t*-butylacetal, and the produced arachidonic acid butyl ester was separated at 200 °C by gas chromatography using a column (3 mm × 2 m) packed with 10% diethylene glycol succinate on Celite 545 (80–100 mesh). The arachidonic acid butyl ester was identified by comparison with its reference standard. Free arachidonic acid was estimated by measuring the ratio of the peak area of arachidonic acid butyl ester to that of tricosanoic acid butyl ester.

Esterified arachidonic acid in synaptosomal lipids was determined as follows [12]. Synaptosome suspension was throughly mixed with 5 vols. of chloroform/methanol (2 : 1, v/v), and the aqueous phase was removed and re-extracted with the same volume of chloroform/methanol (2 : 1, v/v). The organic phases were combined and the combined extracts were evaporated under nitrogen. The dried lipids were dissolved in 100 μl of chloroform and separated into the total phospholipids and the total neutral lipids by thin-layer chromatography on Silica gel 60 in petroleum ether/diethyl ether/acetic acid (85 : 15 : 2, v/v). The zones corresponding to the two lipids were respectively scraped into two tubes and *N,N*-dimethylformamide di-*t*-butylacetal reagent was added after the hydrolysis. The produced butyl esters of fatty acids were determined using the gas chromatographic method as described above.

Preparation of synaptosomes labelled with [^{14}C]-arachidonic acid. Synaptosomes were suspended in a Ca^{2+}-free Ringer's solution to give a concentration of 5 mg of protein per ml. The suspension (5 ml) was incubated with 0.3 μCi of [1-^{14}C]arachidonic acid (5 nmoles) at 37 °C for 30 min. The reaction mixture was cooled to 4 °C and the synaptosomes were sedimented at 23000 × *g* for 10 min.

Extraction and assay of radioactive lipids. Radioactive lipids were extracted from the synaptosomes labelled with [^{14}C]arachidonic acid and the extract was evaporated in the same manner as described above. The dried lipids were dissolved in a small amount of chloroform and spotted on thin-layer chromatographic plate (Silica gel 60). The solvent system consisted of methyl acetate/*n*-propanol/chloroform/methanol/0.25% KCl aqueous solution (25 : 25 : 25 : 10 : 9, v/v). The resolved zones were visualized by means of iodine vapor and were consequently scraped into scintillation vials for assay of radioactivities using a liquid scintillation counter.

Perfusion of synaptosomes. Perfusion experiments were carried out following the method of Raiteri et al. [13] and Hoshino et al. [14]. Synaptosome pellet was suspended in Ca^{2+}-free Ringer's solution to give a protein concentration of 5 mg/ml. The suspension (0.5 ml) was placed on a filter unit that consisted of a 2.4-cm Whatman GF/C and 2.5-cm Millipore filter (pore size 0.45 μm) lying on the bottom of a 20-ml perfusion chamber [14]. The chamber was connected with a peristaltic pump and filled with the Ca^{2+}-free solution at 37 °C. The perfusion rate was 0.5 ml/min, and 2 min fractions were collected into separate tubes. At 20 min (10 fractions) after beginning of perfusion, the Ca^{2+}-free solution was substituted with another solution containing compound to be tested.

Assay of γ-aminobutyric acid. Filtrate or perfusate through the filter unit described above was used as samples to be assayed. The sample (0.5–2.0 ml) was acidified with 0.1 M formic acid to about

pH 4 and passed through a Dowex 50W-X8 column (0.6 × 4 cm). The column was washed with 10 ml of water and γ-aminobutyric acid was eluted with 6 ml of 2 M NH_4OH. γ-Aminobutyric acid in the eluate was measured using the gas chromatographic method of Schmid and Karobath [15], with minor modification [14].

Protein was measured according to Lowry et al. [16].

Results

Effect of Ca^{2+} on release of arachidonic acid in synaptosomes and efflux of γ-aminobutyric acid from them

To examine the effects of Ca^{2+} and depolarizing by high K^+ on release of arachidonic acid, synaptosomes were suspended in Ca^{2+}-free Ringer's solution, and the suspension was added to the same volume of this solution or of Ringer's solution containing 5 mM $CaCl_2$/5 mM KCl/120 mM NaCl, 107 mM KCl/26 mM NaCl, or 5 mM $CaCl_2$/107 mM KCl/18 mM NaCl to give each final concentration as shown in Table I. The suspensions obtained were incubated for 20 s at 37 °C, and at the end of incubation a part of the mixture was mixed with diethyl ether to assay free fatty acids and another part was passed through a filter to assay γ-aminobutyric acid in the medium (see Materials and Methods). Table I shows that the exposure to Ca^{2+}-high K^+ Ringer's solution markedly increased the level of free arachidonic acid, while the presence of Ca^{2+} alone or high K^+ alone had no significant effect on the level. Under these conditions, however, there was no significant increase in any of the other fatty acids. The exposure to Ca^{2+}-high K^+ induced also a clear increase in γ-aminobutyric acid efflux. Accordingly, the effect of Ca^{2+}-high K^+ on arachidonic acid release and γ-aminobutyric acid efflux was investigated at various time intervals using the same method. In the early stage, there were marked increases in arachidonic acid release and γ-aminobutyric acid efflux (Fig. 1). After about 60 s, however, there was recovery to the original level of free arachidonic acid, indicating further metabolism of the acid. The level of γ-aminobutyric acid in the medium remained stationary with the re-

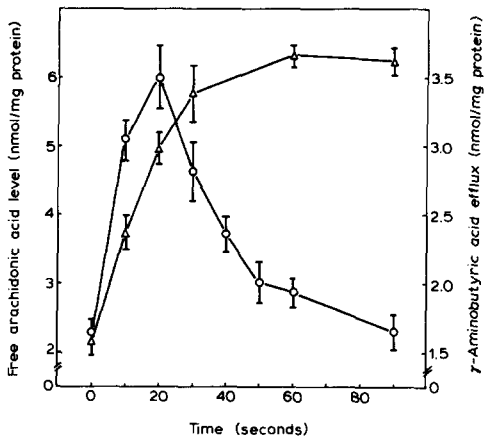

Fig. 1. Changes in free arachidonic acid level and γ-aminobutyric acid efflux with time, after exposure of synaptosomes to Ca^{2+} (2.5 mM) plus high K^+ (56 mM). Each point represents the mean ± S.D. of three experiments. ○, free arachidonic acid level; △, γ-aminobutyric acid efflux.

TABLE I

EFFECT OF Ca^{2+} AND HIGH K^+ ON ARACHIDONIC ACID RELEASE AND γ-AMINOBUTYRIC ACID EFFLUX FROM SYNAPTOSOMES

Values are means ± S.D. for three experiments and are expressed as increases from original levels observed prior to the exposures to the ions. The original levels of free arachidonic acid and γ-aminobutyric acid are 2.228 ± 0.176 and 1.636 ± 0.056 nmol/mg protein, respectively.

	Arachidonic acid release (nmol/20 s per mg protein)	γ-Aminobutyric acid efflux (nmol/20 s per mg protein)
Ca^{2+}-free, low K^+ (5 mM)	0.056 ± 0.232	0.028 ± 0.024
Ca^{2+} (2.5 mM), low K^+ (5 mM)	0.240 ± 0.104	0.052 ± 0.056
Ca^{2+}-free, high K^+ (56 mM)	0.440 ± 0.115	0.380 ± 0.104
Ca^{2+} (2.5 mM), high K^+ (56 mM)	3.780 ± 0.452	1.592 ± 0.104

covery of free arachidonic acid. When synaptosomes pretreated with Ca^{2+} ionophore were exposed to Ca^{2+}, free arachidonic acid was maintained at a high level, and γ-aminobutyric acid efflux also continued with the maintenance of free arachidonic acid, in contrast to the observation of untreated synaptosomes (Fig. 2). The free arachidonic acid released under these conditions was not found in the medium (suspending the synaptosomes), showing that the acid was not liberated into the medium. These results suggest that free arachidonic acid increased by an influx of Ca^{2+} induces γ-aminobutyric acid efflux from synaptosomes.

Origin of arachidonic acid released in synaptosomes

When synaptosomes were incubated at 37°C for 20 s in Ca^{2+}-high K^+ Ringer's solution there was a 14% decrease of arachidonic acid in the phospholipids (Table II), while no significant change was observed in the neutral lipids. The decrease of arachidonic acid (4.96 nmol/mg protein) may sufficiently account for the release of arachidonic acid described above (3.70 nmol/mg protein, see Fig. 1).

To obtain more detail information, synaptosomes were preincubated with [^{14}C]arachidonic acid. This fatty acid is known to be preferentially incorporated to the 2-position of glycerol moiety of phospholipid [17,18]. When the [^{14}C]arachidonic acid-labelled phospholipids were analyzed, 3.7, 2.9, 2.6, and 1.8% of the added [^{14}C]arachidonic acid was incorporated into phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine, respectively. When the synaptosomes were stimulated for 20 s by the Ca^{2+}-high K^+ solution, there was a 15% decrease in the radioactivity of phosphatidylcholine and a 16% decrease of phosphatidylinositol (Table II). There were also slight decreases of phosphatidylserine and phosphatidylethanolamine, but in these cases the decreases were not statistically significant because of the relatively large standard errors. The decreases in arachidonic acid content in phospholipids described above may be accounted for mainly by the release of [^{14}C]arachidonic acid from phosphatidylcholine and phosphatidylinositol.

When synaptosomes were stimulated for 60 s, the arachidonic acid content in phospholipids was recovered to the original level (34.14 nmol/mg protein). On the other hand, the radioactivities of phospholipids were not recovered but slightly decreased, suggesting that phospholipids are resynthesized for 40 s after the first 20 s stimulation possibly using arachidonic acid different from just released one.

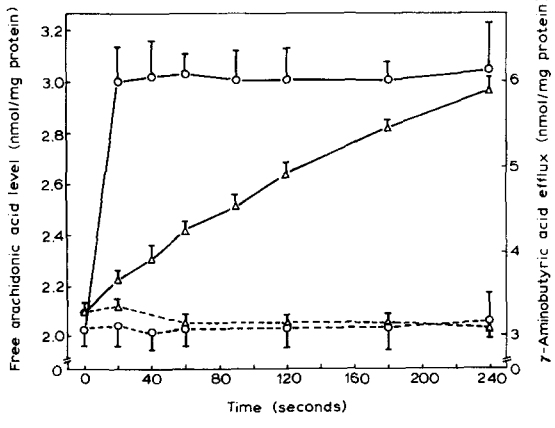

Fig. 2. Changes in free arachidonic acid level and γ-aminobutyric acid efflux with time, after exposure of A23187-treated synaptosomes to Ca^{2+} (2.5 mM). Each point represents the mean ± S.D. of three experiments. ○, free arachidonic acid level; △, γ-aminobutyric acid efflux; ———, in the presence of Ca^{2+}; - - - - - -, in the absence of Ca^{2+}.

Effect of exogenously added arachidonic acid and γ-aminobutyric acid efflux from synaptosomes

To determine whether γ-aminobutyric acid efflux is induced particularly by the arachidonic acid release, the effects of added arachidonic acid and other fatty acids on γ-aminobutyric acid efflux were examined in Ca^{2+}-free Ringer's solution. This experiment was carried out following the perfusion method of Raiteri et al. [13] and Hoshino et al. [14] (see Materials and Methods). After washing with Ca^{2+}-free Ringer's solution, continuous perfusion for 4 min with the solution containing 1.0 mM arachidonic acid caused a large increase in γ-aminobutyric acid efflux (Fig. 3). The maximum efflux, which was about 3-times larger than the baseline (spontaneous) efflux, occurred at 6 min (fraction 3), and then decreased to the level of spontaneous efflux at 16 min (fraction 8). On the contrast, such γ-aminobutyric acid efflux was observed to continue when the synaptosomes were

TABLE II

COMPARISON OF CHANGES IN ARACHIDONIC ACID CONTENT OF SYNAPTOSOMAL PHOSPHOLIPIDS INDUCED BY Ca^{2+}-HIGH K^+

Values are mean ± S.D. for three experiments.

	Stimulation time (s)	Control (1)	Ca^{2+}-high K^+ (2)	Loss (1)−(2)
Arachidonic acid content of phospholipids (nmol/mg protein)				
	0	33.73 ± 1.09		
	20	34.40 ± 1.12	29.44 ± 0.98	4.96 *
	60	33.38 ± 1.33	34.14 ± 1.10	−0.76
Radioactive arachidonic acid of phospholipids (dpm/mg protein)				
Phosphatidylcholine	0	723.2 ±16.2		
	20	725.9 ±22.4	618.3 ±31.6	107.6 *
	60	722.8 ±40.9	584.1 ±18.4	138.7 *
Phosphatidylserine	0	573.4 ±36.9		
	20	570.5 ±11.6	556.5 ±23.9	14.0
	60	569.5 ±34.2	514.3 ±16.5	55.2
Phosphatidylinositol	0	516.1 ±32.0		
	20	516.2 ±11.6	434.9 ±11.4	81.3 *
	60	517.1 ±29.4	405.6 ±27.4	111.5 *
Phosphatidylethanolamine	0	348.6 ±12.4		
	20	345.5 ±46.3	306.7 ±43.1	38.8
	60	337.4 ±12.4	302.3 ±29.8	35.1

* $P < 0.001$ as compared with the control.

successively exposed to 1.0 mM arachidonic acid (Fig. 3). In order to show that the synaptosomes remained intact after this treatment, lactate dehydrogenase (EC 1.1.1.27) activity in the perfusate was assayed. This enzyme should be present in the cytoplasma of synaptosome and could not be found in the perfusate if synaptosomes remained intact. The enzyme activity was little observed in the perfusate even after the addition of arachidonic

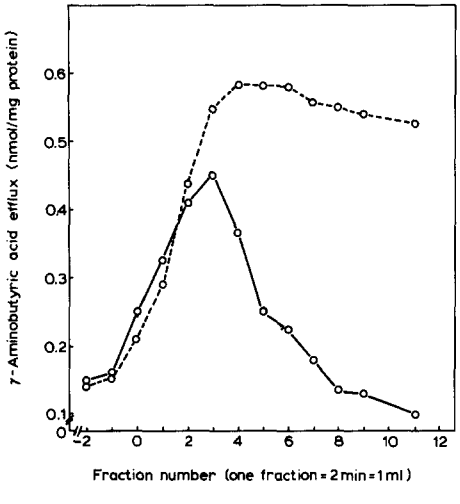

Fig. 3. Effect of exogenously added arachidonic acid on γ-aminobutyric acid efflux from synaptosomes. The exposure to arachidonic acid was started at the fraction of No. 0. O——O, γ-aminobutyric acid efflux by exposure for 4 min to arachidonic acid (1 mM); O- - -O, γ-aminobutyric acid efflux by continuous exposure to arachidonic acid (1 mM). Each point represents the average of two experiments.

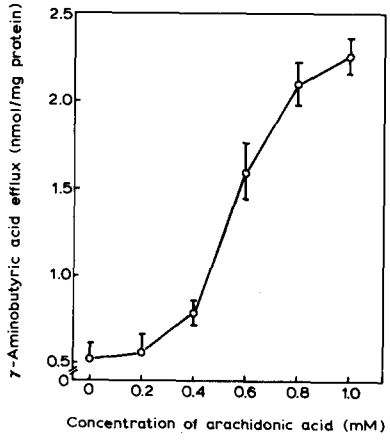

Fig. 4. Effect of different concentrations of arachidonic acid on γ-aminobutyric acid efflux from synaptosomes. The efflux is expressed as γ-aminobutyric acid in perfusate during the 20 min after the start of exposure for 4 min to the fatty acid. Each value represents the mean ± S.D. for three experiments.

TABLE III

EFFECT OF VARIOUS FATTY ACIDS ON γ-AMINOBUTYRIC ACID EFFLUX FROM SYNAPTOSOMES

γ-Aminobutyric acid efflux was determined as described in the text and Fig. 4. The test fatty acids were added at a concentration of 1.0 mM. Values are means ± S.D. for three experiments and are expressed as increases from the baseline efflux (0.492 ± 0.036 nmol/mg protein).

	γ-Aminobutyric acid efflux (nmol/mg protein)
Palmitic acid	0.032 ± 0.056
Stearic acid	0.152 ± 0.104
Oleic acid	0.232 ± 0.072
Linoleic acid	0.240 ± 0.024
Linolenic acid	0.352 ± 0.048
Arachidic acid	0.010 ± 0.076
Arachidonic acid	1.392 ± 0.116
Ca^{2+}-high K^+	1.744 ± 0.116 *

* Shown to compare with fatty acids.

acid (less than 2.5%). Moreover, to examine the effect of various concentrations of arachidonic acid on the γ-aminobutyric acid efflux, perfusate during 20 min after the start of exposure for 4 min was combined and γ-aminobutyric acid in it was assayed. As shown in Fig. 4, about 0.4 mM arachidonic acid substantially enhanced the γ-aminobutiryc acid efflux, and the enhancement reached to a maximal level with about 1 mM arachidonic acid. Furthermore, the effect of other fatty acids than arachidonic acid and of prostaglandins, which were oxygenated products of arachidonic acid, were also examined using the same method. In contrast with arachidonic acid, fatty acids tested had no significant effect on the γ-aminobutyric acid efflux, though a slight effect was found in some unsaturated fatty acids (Table III). Prostaglandins D_2 and E_2 also were almost ineffective (data not shown). These results suggest again that γ-aminobutyric acid efflux from synaptosomes was closely associated with an increase in arachidonic acid concentration in synaptosomes.

Discussion

Previously, we showed that external Ca^{2+} increased γ-aminobutyric acid efflux from synaptosomes when they were depolarized by a high concentration of K^+ or pretreated with a Ca^{2+} ionophore [8]. Data presented in this paper have shown that exposure to Ca^{2+}-high K^+ Ringer's solution markedly increases both arachidonic acid release and γ-aminobutyric acid efflux, while exposure to Ca^{2+} or high K^+ alone has no effect on both (Table I). The exposure to Ca^{2+}-high K^+ solution caused a rapid increase and then a decrease in level of free arachidonic acid within synaptosomes (Fig. 1). The peak in arachidonic acid level was arrived in 20 s after the start of exposure, and the peak concentration in synaptosomes was calculated at about 1.2 mM from the amount of arachidonic acid and the volume per mg protein of synaptosomes [19]. γ-Aminobutyric acid efflux also increased and then decreased in parallel with the accumulation and the recovery of arachidonic acid to original level (Fig. 1). When synaptosomes were pretreated with Ca^{2+} ionophore free arachidonic acid was increased by addition of Ca^{2+} alone and maintained at a high level as long as Ca^{2+} was present in the medium. γ-Aminobutyric acid efflux continued with the maintenance of arachidonic acid at a high level (Fig. 2). These results suggest that the release of arachidonic acid and the efflux of γ-aminobutyric acid are closely associated.

Even in the absence of Ca^{2+}, when fatty acids were exogenously added to synaptosomes, arachidonic acid (but not other fatty acids) increased γ-aminobutyric acid efflux to the same order of potency as that in Ca^{2+}-high K^+ solution (Table III). It is probable that the synaptosomes remained intact after the addition of arachidonic acid, because cytosomal lactate dehydrogenase was not found in the perfusate after this treatment. A maximal stimulation of external arachidonic acid on γ-aminobutyric acid efflux was achieved by increasing the concentration to about 1 mM (Fig. 4), which was nearly similar to the peak concentration (1.2 mM) within synaptosomes when stimulated with Ca^{2+}-high K^+. The added arachidonic acid may be taken up into the synaptosomes without injury to them and then work upon the synaptosomal membrane from within. Moreover, the γ-aminobutyric acid efflux continued as long as the external arachidonic acid was present in the medium (Fig. 3). These results seem likely that the release of arachidonic acid in synaptosomes is one

of metabolic events to occur after Ca^{2+} influx and before the γ-aminobutyric acid efflux response. Moreover, the fact that arachidonic acid released in synaptosomes is rapidly reduced suggests that the arachidonic acid is easily metabolized to some inactive substances. Prostaglandins might be included in such substances. In fact, addition of prostaglandins D_2 and E_2 caused no significant γ-aminobutyric acid efflux (data not shown), though the prostaglandin D_2 is known to depolarize neuroblastoma cells as a model of nerve cells [20].

When synaptosomes labeled with [^{14}C]arachidonic acid were stimulated for 20 s with Ca^{2+}-high K^+ solution, decreases in the radioactivity of phosphatidylcholine and phosphatidylinositol were observed, and the decrease were accompanied by a corresponding decrease in the total arachidonic acid content of phospholipids (Table II). The decrease in arachidonic acid content in phospholipids may quantitatively account for the accumulation of arachidonic acid in synaptosomes as shown in Fig. 1. Free arachidonic acid has been reported to be liberated from the 2-position of phospholipids by at least two kinds of Ca^{2+}-dependent phospholipase reaction, of which one seems to favor the degradation of phosphatidylcholine and the other one that of phosphatidylinositol [21–23]. The present data appear to show the participation of both reactions in the arachidonic acid production. When the exposure to Ca^{2+}-high K^+ solution was continued until 60 s, arachidonic acid content in phospholipids was recovered to the original level, showing that arachidonic acid was again incorporated in the phospholipids. From the results shown in Table II, however, it is presumed that the incorporated arachidonic acid is derived from a different pool of arachidonic acid than that of arachidonic acid just released from phospholipids, and that the just released arachidonic acid is metabolized without being used to synthesize the phospholipids. These metabolic problems remain to be clarified in future experiments. In any case, an apparent correlation between the Ca^{2+} influx, arachidonic acid liberation, and γ-aminobutyric acid efflux shows a possibility that the liberated arachidonic acid acts as an intermediator between the Ca^{2+} influx into synaptosomes and the γ-aminobutyric acid efflux from them. The inflowed Ca^{2+} may be a trigger to cause a rapid increase and recovery in level of arachidonic acid produced from phospholipids, and the alteration of arachidonic acid level may be associated with the process of opening and shutting path-ways for γ-aminobutyric acid efflux.

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