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INVOLVEMENT OF PENTYLENETETRAZOLE IN SYNAPSIN I PHOSPHORYLATION ASSOCIATED WITH CALCIUM INFLUX IN SYNAPTOSOMES FROM RAT CEREBRAL CORTEX

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Abstract—To determine precisely how pentylenetetrazole (PTZ) is involved in the biochemical processes at the presynaptic nerve terminal, the effect of PTZ, under various conditions, on the phosphorylation of synapsin I (previously called protein I) was investigated, using $^{32}P_i$ in synaptosomes from rat cerebral cortex. PTZ markedly stimulated the incorporation of ^{32}P into this protein as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and autoradiography, but it failed to stimulate protein phosphorylation in Ca^{2+}-free medium containing ethylene glycol bis-(β-aminoethylether)-*N*′,*N*′-tetraacetic acid (EGTA). Moreover, the PTZ-stimulated synapsin I phosphorylation was reversed by addition of EGTA sufficient to chelate all external free Ca^{2+}. PTZ also stimulated synaptosomal accumulation of Ca^{2+}. The PTZ-stimulatory effects of both synapsin I phosphorylation and synaptosomal accumulation of Ca^{2+} were inhibited markedly by tetrodotoxin as well as by cobalt chloride and lanthanum chloride. The calmodulin antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, strongly) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5, weakly) reduced the PTZ-stimulatory effect on synapsin I phosphorylation by about 75 and 15%, respectively, whereas these antagonists had essentially no effect on PTZ-stimulated synaptosomal accumulation of Ca^{2+}. These results suggest that PTZ causes the influx of Ca^{2+} into the presynaptic nerve terminal secondary to the elevated Na^+ and is consequently involved in the synapsin I phosphorylation step, facilitating the Ca^{2+}/calmodulin-mediated presynaptic event leading to seizure discharge.

One experimental approach in the attempt to understand the neurophysiological basis of epileptic seizures has been to study the action of convulsant drugs [1, 2]. The convulsant drug pentylenetetrazole (PTZ§) has been studied extensively in invertebrate preparations in order to determine the mechanism of epileptogenesis [3–15], because seizure discharge induced by this drug in invertebrates shows neuronal activity very similar to the paroxysmal depolarization shift observed in epileptic cortical neurons [16, 17]. Although the role of PTZ is not yet definitive, three possible mechanisms by which it acts at the cellular level have been proposed. One possibility is that PTZ acts as a selective neutralizer of γ-aminobutyric acid by removing its postsynaptic inhibitory effect [2–5]. Another possibility is that PTZ affects membrane properties, thus increasing spontaneous discharge by altering ionic conductance [6–9]. A third possibility is that PTZ plays a role in intracellular Ca^{2+}-related cytoplasmic reactions [9–15]. However, essentially no evidence has been presented to demonstrate a role for PTZ at the presynaptic nerve terminal.

It has been generally accepted that influx of Ca^{2+} into the terminal provides a depolarization-induced trigger for the release of neurotransmitter molecules [18, 19] and promotes the synthesis of neurotransmitter substance [20, 21]. Forn [22] and Browning *et al.* [23] indicate that many of the Ca^{2+}-elicited responses observed in vertebrate tissues reflect the influence of Ca^{2+} on protein phosphorylation. Their model proposes that a voltage-dependent increase in the level of Ca^{2+} in the nerve terminal activates the actual response as a second messenger through endogenous protein kinase and the resultant phosphorylation of some synaptic proteins which then affect neuronal events by changing the ionic permeability of the junctional membrane. Conditions under which Ca^{2+} transport across the plasma membrane at the terminal is increased lead to an increase in phosphorylation of several synaptosomal proteins [24–28]. Also, release of norepinephrine from synaptic vesicles has been shown to correlate with the Ca^{2+}-activated phosphorylation of specific endogenous proteins [28, 29].

Therefore, if the effect of PTZ on protein phosphorylation associated with Ca^{2+} influx is examined in synaptosomes, the chain of PTZ-induced intracellular events at the presynaptic nerve terminal which produces an overly excitable neuronal popu-

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§ Abbreviations: PTZ, pentylenetetrazole; EGTA, ethylene glycol bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; KRB, Krebs–Ringer buffer; and H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide.

lation, leading to seizure discharge, will be defined, considering the fact that excessive amounts of Ca^{2+} enter the nerve terminal during and following this discharge [30–32]. In the present paper, we describe the effect of PTZ on protein phosphorylation associated with Ca^{2+} influx in synaptosomes from rat cerebral cortex. It was found that PTZ enhanced synaptosomal uptake of Ca^{2+} and phosphorylation of synapsin I in a Ca^{2+}/calmodulin-dependent manner.

MATERIALS AND METHODS

Materials. PTZ and tetrodotoxin (TTX) were obtained from the Sigma Chemical Co.; H-8, W-7 and W-5 from the Seikagaku Kogyo Co.; calcium ionophore A23187 from Calbiochem-Behring; $^{32}P_i$ (63 mCi/ml carrier-free orthophosphate) and $^{45}CaCl_2$ (39.4 mCi/mg Ca^{2+}) from New England Nuclear; ACS scintillator from Amersham/Searle; and molecular weight protein standards from Boehringer Mannheim. All other chemicals were of analytical grade.

Crude synaptosomes. Crude synaptosomes were prepared by a method based on that of Whittaker [33]. Adult male Wistar rats (200–250 g) were killed by decapitation; the brain was removed rapidly and the cerebral cortex was separated from the white matter. The cerebral cortex was homogenized in ice-cold 0.32 M sucrose solution (pH 7.4) in a Teflon–glass homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min, and the resultant supernatant fraction was recentrifuged at 15,000 *g* for 30 min. The pellet was then washed by resuspending in sucrose solution followed by centrifugation at the same centrifugal force. The pellet (P2) obtained was used for subsequent experiments except where indicated.

Subfractionation of crude synaptosomes. Subfractionation of the P2 was achieved essentially by the method of Gurd *et al.* [34]. P2 from five rats was suspended in the above sucrose solution. This was then diluted with 19% Ficol (w/v) dissolved in sucrose solution to give a final Ficol concentration of about 14%. A discontinuous density gradient was made by overlaying 15 ml of this suspension, first with 10 ml of 7.5% (w/v) Ficol in sucrose solution, and then with 5 ml of sucrose solution alone. This gradient was then centrifuged for 120 min at 90,000 *g* in a Hitachi 65P swinging-bucket rotor. The material both at the 0 to 7.5% Ficol interface (fraction A) and the 7.5 to 14% Ficol interface (fraction B) was harvested with a Pasteur pipette. The pellet was fraction C. Each of these fractions, as well as that portion of the P2 fraction in 14% Ficol which had not been subjected to centrifugation, was diluted to 50 ml with sucrose solution and centrifuged at 20,000 *g* for 10 min. A part of each resultant pellet was fixed, dehydrated, embedded, and sliced into thin sections which were examined with a JEM 200 CX electron microscope.

Phosphorylation, gel electrophoresis, autoradiography, microdensitometry and molecular-weight determination. Phosphorylation of synaptosomes was carried out as described previously [24]. Briefly, a sample of the P2 fraction, suspended in Krebs–Ringer buffer (KRB) containing 0.1 mM EGTA, was prelabeled with 0.2 mCi/ml $^{32}P_i$ for 30 min at 37°. Following prelabeling, 0.2-ml aliquots of synaptosomal suspension were added to test tubes containing 20 mM PTZ in 0.2 ml KRB. These tubes also contained 2.1 mM Ca^{2+} (2.2 mM added $CaCl_2$) except where indicated. Incubation was continued for 5 min (unless otherwise specified). Reaction was terminated by addition of 0.2 ml of 10% (w/v) SDS, and the incubation tubes were placed in boiling water for 2 min. Forty microliters of a solution containing Tris–HCl (500 mM, pH 7.4), β-mercaptoethanol (25 mM), sucrose (0.5 g/ml) and bromphenol blue (0.3 mg/ml) was added, and the tubes were kept overnight at room temperature. Proteins (50 μg/lane) were resolved by 10% polyacrylamide SDS-slab gel electrophoresis as in the method of Laemmli and Favre [35]. Gels were fixed and stained with 0.1% (w/v) Coomassie brilliant blue R, 50% (v/v) methanol, and 7% (v/v) acetic acid solution before being dried under heat at reduced pressure with an Atto slab-gel drier. Autoradiography was carried out for 4–6 days with Kodak X-Omat RP film. Incorporation of ^{32}P into specific protein bands was estimated by microdensitometry of the autoradiograph with a Joese–Loeble spectrophotometer. Protein phosphorylation was quantified by direct counting of bands as well as by the method in Ref. 24. The apparent subunit molecular weight of the phosphoprotein was determined by comparison with the mobility of proteins of known molecular weight.

Ca^{2+} uptake into synaptosomes. Ca^{2+} uptake into synaptosomes was determined essentially as described in Ref. 24. The P2 suspension was preincubated in KRB containing 1 mM Ca^{2+} at 37° for 5 min. Aliquots (0.2 ml) of the suspension were then added to test tubes containing test agents and 0.1 μCi of $^{45}CaCl_2$ in 0.2 ml KRB containing 1 mM Ca^{2+} and incubated for another 5 min except where indicated. After incubation, 5 ml of ice-cold KRB without Ca^{2+} was added to each tube. Using a membrane apparatus, this suspension was rapidly filtered on a Whatman GC filter which was rinsed three times with 5 ml of the same ice-cold KRB. Filters were transferred into scintillation vials containing 1 ml of 1% (w/v) SDS and allowed to stand for 20 min before adding 12 ml of ACS scintillation fluid. Radioactivity was determined by a scintillation counter (Aloka liquid scintillation system, LSC-753). "$^{45}Ca^{2+}$ uptake", which was calculated from the $^{45}Ca^{2+}$ remaining on the filters, was used as a rough estimate of the amount of Ca^{2+} accumulated by the synaptosomes. $^{45}Ca^{2+}$ uptake was expressed as nmoles/mg protein.

Protein assay. Protein was assayed by the method of Hartree [36] with bovine serum albumin as standard.

RESULTS

An autoradiograph showing the effect of 10 mM PTZ on the incorporation of ^{32}P into proteins of a crude synaptosomal fraction (P2) from rat cerebral cortex is shown in Fig. 1. Approximately 30–50 individual protein bands were revealed by protein staining of the gel. Of these protein bands, incorporation of ^{32}P into a protein doublet (synapsin I) [$\bar{X} \pm$ SE control, 100 (lane 1); PTZ, 202.4 ± 10.8 (lane 2,

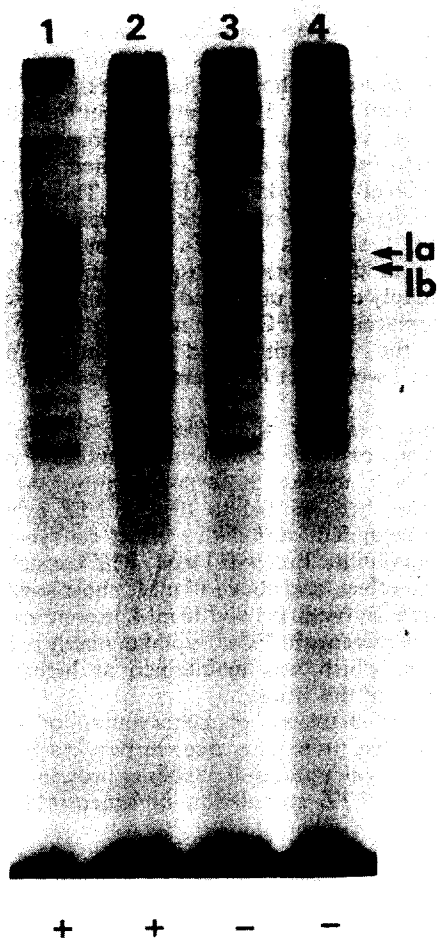

Fig. 1. Effect of PTZ in the absence (−) or presence (+) of Ca^{2+} on phosphorylation of endogenous proteins in crude synaptosomal preparations. After preincubation with $^{32}P_i$ for 30 min, aliquots were incubated for 5 min in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 mM PTZ. Incubation was terminated by addition of SDS, and samples were subjected to SDS–polyacrylamide gel electrophoresis; gels were stained, dried, and exposed to X-ray film for autoradiography. Arrows indicate synapsins Ia and Ib.

$P < 0.001$, $N = 6$)], that migrated at 86 and 80 kD as described [24], was enhanced markedly when the synaptosome suspension was incubated with 10 mM PTZ and 1 mM Ca^{2+} for 5 min. PTZ slightly stimulated, in addition, phosphorylation of a few bands in the 50- to 70-kD range. Bands of approximately 95 kD showed reduced ^{32}P incorporation. In contrast, PTZ did not effect a change in the phosphorylation in the absence of Ca^{2+} (compare lanes 3 and 4). However, we discuss here only synapsin I phosphorylation.

In the other experiments we directly measured the ^{32}P content in synapsin I using a scintillation counter. In the presence of Ca^{2+} ($N = 6$), the ^{32}P in synapsin I in the PTZ was enhanced to nearly two times when compared with that in synapsin I in the control ($\bar{X} \pm$ SE control, 19,507 ± 1,637 dpm; PTZ, 37,633 ± 2,688 dpm, $P < 0.001$). In contrast, in the absence of Ca^{2+} ($N = 6$), there was no difference between radioactivity in this protein in the control and that in the PTZ ($\bar{X} \pm$ SE control, 17,968 ± 1,680 dpm; PTZ, 18,549 ± 1,922 dpm). These results were almost identical with those obtained by the autoradiographic method which was used in subsequent phosphorylation experiments.

In order to strengthen the evidence that the phosphoprotein doublet is synapsin I, this protein band was out of the gel and subjected to limited proteolysis during SDS–polyacrylamide gel electrophoresis, as described in Ref. 37. In agreement with the previous findings on synapsin I [37], autoradiography of the gel revealed an undigested protein doublet at the top of the gel, together with an upper peptide fragment of approximately 35 kD and a lower peptide fragment of approximately 10 kD (data not shown). We, therefore, concluded that the protein band described as synapsin I is, in fact, synapsin I.

To confirm that PTZ-stimulated synapsin I phosphorylation was associated with the influxed Ca^{2+} through the synaptosomal membrane, we compared the time course of $^{45}Ca^{2+}$ uptake with that of synapsin I phosphorylation in the presence of 10 mM PTZ. $^{45}Ca^{2+}$ uptake in the crude synaptosomal preparation occurred in the absence and to a greater extent in the presence, of PTZ, reaching a maximum level 10–15 min after addition of PTZ and $^{45}Ca^{2+}$ (Fig. 2A). It was observed in the present investigation that the time course of $^{45}Ca^{2+}$ uptake in the absence of PTZ was independent of the preincubation time (5–25 min) in KRB containing 1 mM unlabeled Ca^{2+} (data not shown), which indicates that the uptake in samples without PTZ is due to exchange of labeled and unlabeled Ca^{2+} rather than to net accumulation of Ca^{2+} in synaptosomes. As the difference between $^{45}Ca^{2+}$ uptake in the absence or presence of PTZ probably represents PTZ stimulation, the difference in $^{45}Ca^{2+}$ uptake from the control sample is hereafter expressed as "stimulated $^{45}Ca^{2+}$ uptake". This Ca^{2+} uptake increased sharply within 5 min and increased by nearly 100% in 15 min (broken curve, Fig. 2A). In contrast, in the presence of PTZ, synapsin I phosphorylation (Fig. 2B) reached the maximum level within 5 min and decreased slowly thereafter; in the absence of PTZ, there was no significant increase. These results indicate that most of the Ca^{2+} entered the synaptosomes within the first 5 min, in time to stimulate synapsin I phosphorylation.

That stimulation of synapsin I phosphorylation of PTZ was due to the increased Ca^{2+} influxed through the synaptosomal membrane was also supported by the observation of Ca^{2+} uptake and synapsin I phosphorylation in the presence of calcium ionophore A23187. Figure 3 shows an increase in stimulated uptake of $^{45}Ca^{2+}$ with this ionophore which is only half that with PTZ and an increase in protein phosphorylation which is very close to that with PTZ. There was no increase in synapsin I phosphorylation with A23187 when Ca^{2+} was omitted from the incubation medium (data not shown). Response time with the ionophore, however, was nearly 50% slow than that with PTZ in both cases.

If PTZ-stimulated phosphorylation of synapsin I is mostly dependent on the influxed Ca^{2+}, it should

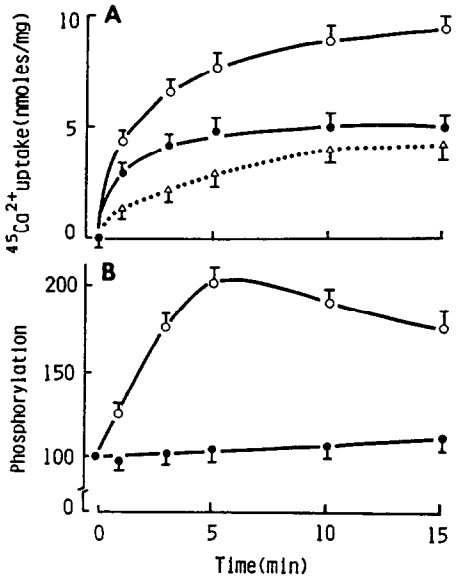

Fig. 2. Time course of PTZ-stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation in a crude synaptosomal preparation. (A) $^{45}Ca^{2+}$ uptake: the fraction P2 had been preincubated for 5 min in KRB containing 1 mM Ca^{2+} prior to incubation with 10 mM PTZ. At zero time, $^{45}CaCl_2$ (●) or PTZ and $^{45}CaCl_2$ (○) were added. The difference between $^{45}Ca^{2+}$ uptake in the absence or presence of PTZ is shown by the broken curve (△). (B) Synapsin I phosphorylation: after preincubation with $^{32}P_i$ for 30 min, aliquots were incubated with 10 mM PTZ. The resultant autoradiograph was scanned by densitometry, and the peak area for phospho-synapsin I was determined. Data are expressed as 100× peak area of phospho-synapsin I in the PTZ/peak area of phospho-synapsin I at zero time in the control (without PTZ). Each point represents the mean of quadruplicate determinations and each bar, SE.

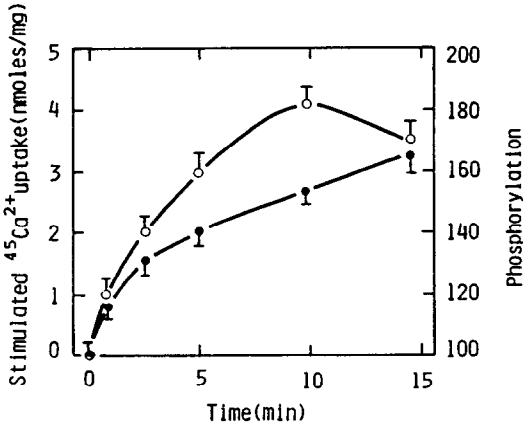

Fig. 3. Time course of the stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation in the presence of 30 μM calcium ionophore A23187 in a crude synaptosomal preparation. $^{45}Ca^{2+}$ uptake (●): as described in the legend in Fig. 2, $^{45}CaCl_2$ or A23187 and $^{45}CaCl_2$ were added at zero time. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from the control sample (without A23187). Synapsin I phosphorylation (○): data are expressed at 100× peak area of phospho-synapsin I in the A23187/peak area of phospho-synapsin I at zero time in the control. Each point represents the mean of quadruplicate determinations and each bar, SE.

be reversed by removal of external Ca^{2+}. As expected, when EGTA was added to chelate free Ca^{2+}, synapsin I phosphorylation returned to the control level within 5 min, whereas in the absence of EGTA, synapsin I phosphorylation diminished about half-way from its PTZ-stimulated level to its control level within 15 min (Fig. 4). This complete reversibility of PTZ-stimulated phosphorylation of synapsin I suggests that Ca^{2+} stimulates a true increase in the protein phosphorylation. Thus, if Ca^{2+} simply caused unlabeled synapsin I phosphate to be replaced by $^{32}PO_4$ with no change in net phosphate, the PTZ-stimulated phosphorylation of synapsin I would not be reversible upon removal of Ca^{2+}.

Figure 5 shows the effects of various concentrations of PTZ on synapsin I phosphorylation and $^{45}Ca^{2+}$ uptake in synaptosomes. At concentrations between 0.1 to 10 mM, PTZ exhibited a dose-dependent stimulative effect on synapsin I phosphorylation. Below 0.1 mM, PTZ caused practically no effect, and above 10 mM, phosphorylation decreased. Between 0.1 and 30 mM, in contrast, PTZ caused an increase in $^{45}Ca^{2+}$ uptake, nearly reaching a plateau which was maintained at higher concentration of this drug.

The Ca^{2+}-activated phosphorylation of specific synaptosomal proteins is due, in part, to the activation of calmodulin-dependent protein kinase [23, 29]. If PTZ stimulation of synapsin I phos-

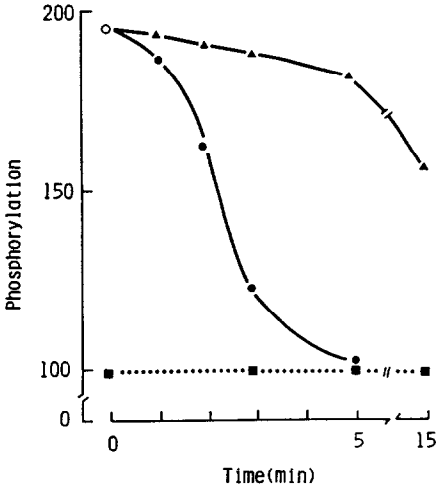

Fig. 4. Effect of removal of Ca^{2+} on PTZ-stimulated synapsin I phosphorylation. The crude synaptosomal preparation was preincubated with $^{32}P_i$ in KRB containing 0.2 mM $CaCl_2$ (0.1 mM free Ca^{2+}) for 30 min in the absence and then for 5 min in the presence of 10 mM PTZ. At the end of this preincubation period (○), EGTA (final concentration, 1.5 mM) was added to some (●) but not to other (▲) aliquots of the suspension. After incubation for the indicated times, the samples were analyzed for ^{32}P in synapsin I. Synapsin I phosphorylation was unchanged in the absence of PTZ (■) and EGTA did not affect this basal level of synapsin I phosphorylation. Data are expressed as 100× peak area for phospho-synapsin I in the treatment/peak area for phospho-synapsin I after a 5-min incubation in KRB. This experiment was repeated three times with similar results.

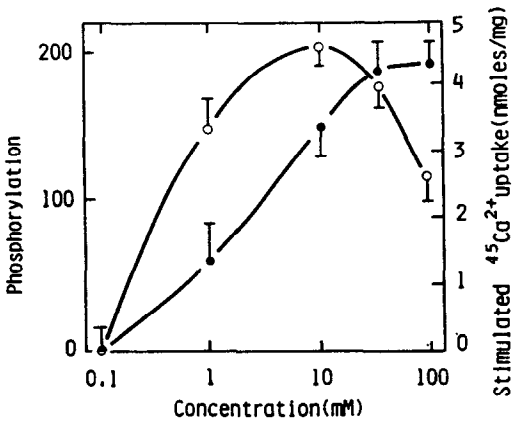

Fig. 5. Effect of PTZ concentration on $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation by synaptosomes. $^{45}Ca^{2+}$ uptake (●): after preincubation for 5 min in KRB containing 1 mM Ca^{2+}, incubation of P2 with $^{45}CaCl_2$ was carried out for 5 min without or with PTZ. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from the control sample (without PTZ). Synapsin I phosphorylation (○): after preincubation with $^{32}P_i$ for 30 min, aliquots were incubated for 5 min without or with PTZ. Data are expressed as 100× peak area of phospho-synapsin I in the PTZ/peak area of phospho-synapsin I in the control. Each point represents the mean of quadruplicate determinations and each bar, SE.

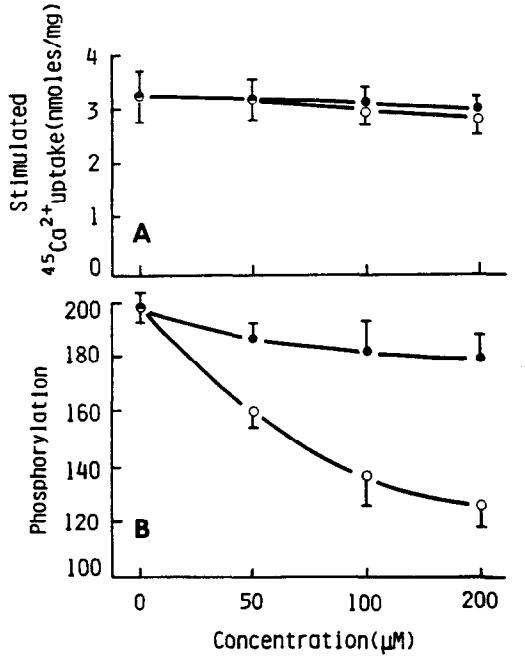

Fig. 6. Comparison of the effects of calmodulin antagonists, W-5 (●) and W-7 (○) on PTZ-stimulated $^{45}Ca^{2+}$ uptake (A) and synapsin I phosphorylation (B) in crude synaptosomal preparations. After preincubation, aliquots were incubated for 5 min in KRB containing 10 mM PTZ and W-5 or W-7. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from the control sample (KRB). Phosphorylation data are expressed as 100 × peak area of phospho-synapsin I in the PTZ/peak area of phospho-synapsin I in the control. Each point represents the mean of quadruplicate determinations and each bar, SE.

phorylation is mediated through Ca^{2+}/calmodulin-dependent protein kinase, involvement of calmodulin in such phosphorylation should be demonstrable by the difference between the inhibitory effect of a strong (W-7) vs a weak (W-5) calmodulin antagonists [38, 39]. Figure 6A shows essentially no effect with either W-5 or W-7 on PTZ-stimulated $^{45}Ca^{2+}$ uptake. Synapsin I phosphorylation was decreased maximally by about 15% with W-5 (Fig. 6B). In contrast, W-7 reduced this protein phosphorylation by about 75% in a concentration-dependent manner.

Since depolarization of synaptosomes causes the Ca^{2+}-dependent activation of cyclic AMP-dependent protein kinase [40], we also examined the effect of H-8 [41], a cyclic AMP-dependent protein kinase inhibitor, on PTZ-stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation. No effect of H-8 on PTZ-stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation was observed (Fig. 7).

To gain some clue of the mechanisms of PTZ promotion of the Ca^{2+} influx, leading to increase in synapsin I phosphorylation, the effects of PTZ on $^{45}Ca^{2+}$ uptake and on synapsin I phosphorylation were tested in the presence of cobalt chloride or lanthanum chloride, each of which has been shown to selectively inhibit Ca^{2+} channels in the cell membrane [42], or in the presence of tetrodotoxin (TTX) which is an Na^+ channel blocker [42]. As shown in Fig. 8, both of these Ca^{2+} channel blockers reduced PTZ-stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation by about 50 and 60%, respectively, whereas incubation in KRB containing blocker but no PTZ produced results very close to those of pre-PTZ incubation. TTX also suppressed $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation by about 66 and 80% respectively (Fig. 9).

The crude synaptosomal fraction (P2) consists of a variety of subcellular organelles [43]. We purified

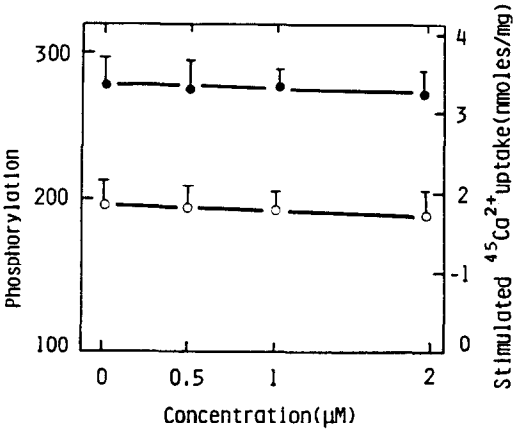

Fig. 7. Effect of H-8 on PTZ-stimulated $^{45}Ca^{2+}$ uptake (●) and synapsin I phosphorylation (○) in crude synaptosomal preparation. After preincubation, aliquots were incubated for 5 min in KRB containing 10 mM PTZ and H-8. The stimulated $^{45}Ca^{2-}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from the control sample (KRB). Phosphorylation data are expressed as 100 × peak area for phospho-synapsin I in the treatment/peak area for phospho-synapsin I in the control. Each point represents the mean of quadruplicate determinations and each bar, SE.

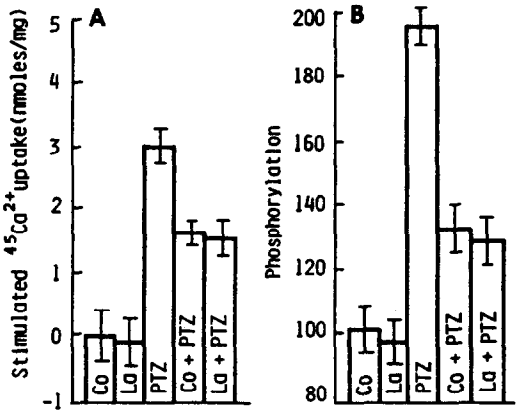

Fig. 8. Effects of Ca^{2+} channel blockers, cobalt chloride or lanthanum chloride, on PTZ-stimulated $^{45}Ca^{2+}$ uptake (A) and synapsin I phosphorylation (B) in a crude synaptosomal preparation. After preincubation, aliquots were incubated for 5 min under the conditions indicated. The final concentrations of the additions were: PTZ, 10 mM; cobalt chloride, 10 mM; and lanthanum chloride, 5 mM. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from that of the control sample (KRB). Phosphorylation data are expressed as 100 × peak area for phospho-synapsin I under conditions indicated/peak area for phosphosynapsin I in the control. Values are the means ± SE of quadruplicate determinations.

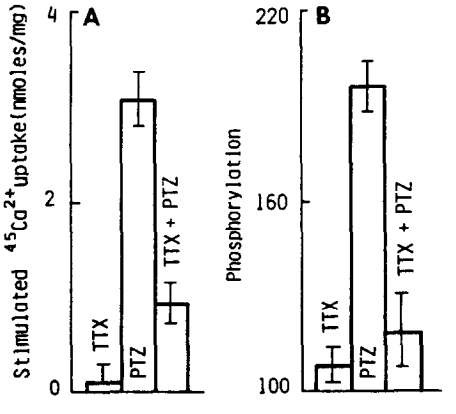

Fig. 9. Effect of TTX on PTZ-stimulated $^{45}Ca^{2+}$ uptake (A) and synapsin I phosphorylation (B) in a crude synaptosomal preparation. After preincubation, aliquots were incubated for 5 min under the conditions indicated. The final concentrations of PTZ and TTX were 10 mM and 10 μM respectively. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from that of the control sample (KRB). Phosphorylation data are expressed as 100 × peak area for phospho-synapsin I under conditions indicated/peak area for phosphosynapsin I in the control. Values are the means ± SE of quadruplicate determinations.

the P2 in order to further pinpoint synaptosomes as the site in which the PTZ effect on synapsin I phosphorylation occurs. The P2 was subfractioned as described in Materials and Methods. We collected the 7.5 to 14% Ficol interface (fraction B) and examined it by electron microscopy. Figure 10 shows the ultrastructure of P2 and fraction B. In agreement with previously published studies [33, 43], P2 contained myelin, membrane fragments, synaptosomes and mitochondria. Fraction B contained a high concentration of synaptosomes with some myelin material. Effects of PTZ on $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation were compared between P2 and fraction B. As shown in Fig. 11, there was no difference in Ca^{2+} uptake and synapsin I phosphorylation between P2 and fraction B. Thus, it appears that most of the PTZ-stimulated $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation observed in P2 is associated with synaptosomes rather than with other subcellular organelles.

DISCUSSION

The present study was undertaken to gain clues as to the mechanism of PTZ action on presynaptic nerve terminals in rat cerebral cortex synaptosomes. Our results show that PTZ, in association with Ca^{2+} influx, caused a marked increase of synapsin I phosphorylation in intact synaptosomes.

Strömbom *et al.* [44] reported that, like the present *in vitro* findings, intraperitoneal administration of PTZ stimulated synapsin I phosphorylation, whereas addition of PTZ (about 0.4 mM) to brain homogenate at a concentration greater than those expected in the brain after its *in vivo* administration did not affect this protein phosphorylation. This means that the effect of PTZ on synapsin I phosphorylation *in vivo* could be brought about by activation of a cyclic AMP-dependent protein kinase rather than by that of a Ca^{2+}-dependent protein kinase, considering the facts that synapsin I is phosphorylated only by Ca^{2+} influx in intact synaptosomes [22, 24, 37]: that it is also located in the postsynaptic regions [22, 45]; and that this protein phosphorylation is stimulated by incubation of the brain slices with cyclic AMP, cyclic AMP derivatives, or phosphodiesterase inhibitor but in a Ca^{2+}-independent manner [45]. Our new finding is that higher concentrations of PTZ than 1 mM stimulated synapsin I phosphorylation in a Ca^{2+}-dependent manner.

Synapsin I has been proposed as primarily a presynaptic protein which is phosphorylated by a cyclic AMP-dependent protein kinase and by a Ca^{2+}-calmodulin-dependent protein kinase [22, 23, 25, 46–49]. The protein whose phosphorylation was enhanced by PTZ also exhibited the stimulatory effect on the phosphorylation in the presence of cyclic AMP in lysed synaptosomes but not in intact synaptosomes,* which was similar to the previous observations on synapsin I phosphorylation [24, 44, 46].

In the present experiments, PTZ stimulated synapsin I phosphorylation to a maximum level within 5 min and dephosphorylation occurred slowly thereafter. This pattern is comparable to Ca^{2+}-dependent phosphorylation of this protein produced with depolarization of synaptosomal membrane by high K^+ or veratridine, although the time courses were somewhat different [24–27]. In addition, synapsin I phosphorylation was reversible upon the removal of Ca^{2+} or was reduced by Ca^{2+} channel blockers (cobalt chloride or lanthanum chloride). These results sug-

* M. Onozuka and S. Imai, unpublished observations.

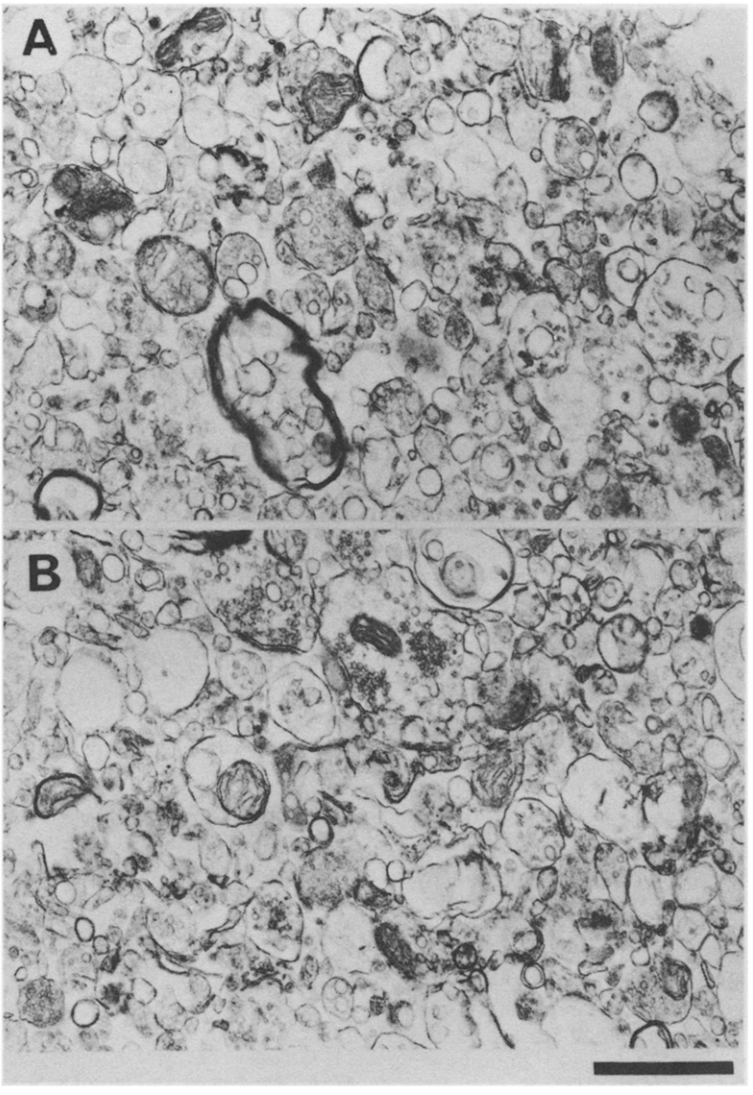

Fig. 10. Electron micrographs of P2 (A) and fraction B (B). Bar indicates 1 μm.

gest that PTZ promotes the influx of Ca^{2+} through the synaptosomal membrane. This assumption is also supported by the observation that the calcium ionophore A23187 mimicked the effect of PTZ on the state of synapsin I phosphorylation.

The question is how does PTZ promote the influx of Ca^{2+} into the synaptosomes. There are two possible answers: first, PTZ may cause a direct activation of the Ca^{2+} channels; and second, it may cause a prior ion event which is followed by an increase in Ca^{2+} permeability of the synaptosomal membrane. When synaptosomes labeled with $^{32}P_i$ were incubated in the presence of PTZ and TTX, both the stimulated uptake of Ca^{2+} and the increase in synapsin I phosphorylation were reduced nearly to the pre-PTZ level. Also, Krueger *et al.* [24] and DeLorenzo *et al.* [50] demonstrated that TTX blocks stimulation of both Ca^{2+} uptake and subsequent protein phosphorylation in synaptosomes by veratridine or scorpion venom. Previously, we found that either the calcium ionophore A23187 (50 μM) [15] or veratridine (1–10 μM) [51] mimicked PTZ-induced seizure discharge. The latter agent also mimicks PTZ-induced development of a negative slope resistance in the current-voltage curve for which sodium ions are a main charge carrier [51]. Therefore, although our observations do not rule out completely the possibility that increase in Ca^{2+} influx into synaptosomes is independent of intrasynaptosomal Na^+, we propose that increased intrasynaptosomal Ca^{2+} during exposure to PTZ is secondary to an elevated intrasynaptosomal [Na^+] which consequently leads to depolarization of the synaptosomal membrane.

Kanamori *et al.* [38] and Hidaka *et al.* [39] showed that W-5 and W-7 inhibit calmodulin's biological activity by their selective binding to a Ca^{2+}/calmodulin complex, and that the action of the former is less specific than that of the latter. Based on the affinities of the antagonist to calmodulin, the difference between the inhibition of PTZ-stimulated synapsin I phosphorylation by W-7 and that by W-5 indicates that the PTZ-induced effect on synapsin I phosphorylation involves Ca^{2+}/calmodulin-depen-

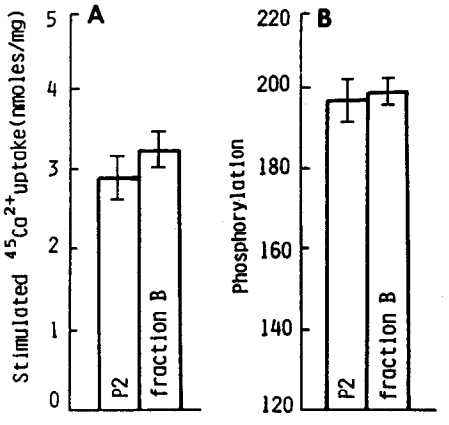

Fig. 11. Effects of PTZ on $^{45}Ca^{2+}$ uptake (A) and synapsin I phosphorylation (B) in crude (P2) and in purified (fraction B) synaptosomal preparations. Fraction B was obtained by subfractionation on a discontinuous Ficol/sucrose flotation gradient. $^{45}Ca^{2+}$ uptake and synapsin I phosphorylation were determined after incubation in the presence of 10 mM PTZ for 5 min. The stimulated $^{45}Ca^{2+}$ uptake indicates the difference in $^{45}Ca^{2+}$ uptake from the control sample (KRB). Synapsin I phosphorylation was determined in the same amount of protein from each fraction (50 μg/lane). Phosphorylation data are expressed as 100 × peak area for phospho-synapsin I in the PTZ/peak area for phosphosynapsin I in the control. Values are the mean ± SE of quadruplicate determinations.

dent protein kinase. Similar results were also observed with the calcium ionophore A23187.* Therefore, PTZ appears to stimulate Ca^{2+}/calmodulin-mediated biochemical events at the presynaptic nerve terminal. The role of Ca^{2+}/calmodulin-dependent phosphorylation of synapsin I has been implicated in the dissociation of this protein from the vesicles, thus overcoming the constraint on neurotransmitter release [22, 23]. Our findings using the calmodulin antagonists also provide a concrete mechanism for the entry of an excessive amount of Ca^{2+} into the nerve terminal during and following epileptoform discharge [30–32]. In *Euhadra* neurons, in addition, PTZ enhances phosphorylation of 34- and 50-kD proteins, and W-7 completely abolishes both PTZ-induced seizure discharge and the PTZ-stimulatory effect on phosphorylation of these two proteins [15], suggesting that PTZ action is generated through Ca^{2+}/calmodulin-dependent protein phosphorylation.

Recently, Dunkley *et al.* [40] indicated that the Ca^{2+} increased during depolarization causes the activation of cyclic AMP-dependent protein kinase in intact synaptosomes as well as Ca^{2+}-dependent protein kinase. However, it appears that stimulation of synapsin I phosphorylation by PTZ was not brought about by this process, judging from the observation obtained by the a cyclic AMP-dependent protein kinase inhibitor.

From the above findings, we suggest the following molecular mechanism of PTZ action at the presynaptic nerve terminal: PTZ causes increase in Na^+ conductance and consequent membrane depolarization which promotes the influx of Ca^{2+} into the presynaptic nerve terminal. The increased Ca^{2+} activates calmodulin and subsequently leads to Ca^{2+}/calmodulin-dependent protein phosphorylation which changes normal synaptic activity to the overstimulation of numerous synaptic contacts, resulting in seizure activity.

* S. Imai and M. Onozuka, manuscript submitted for publication.

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