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Research Article

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Evidence that Parathyroid Hormone-mediated Calcium Transport in Rat Brain Synaptosomes Is Independent of Cyclic Adenosine Monophosphate

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Abstract

In vivo PTH administration to rats resulted in increased brain synaptosomal Ca^{++} transport, while parathyroidectomy (PTX) resulted in decreased transport. To determine the mechanism of action of PTH on Ca^{++} transport in rat brain synaptosomes, we performed transport studies by the Na-Ca exchanger and also measured cAMP generation in synaptosomes from PTX rats. Ca^{++} transport was studied after in vivo additions of either bovine (b)PTH, cAMP, or forskolin, and adenylate cyclase activity was assessed after additions of either bPTH, forskolin, sodium fluoride (NaF), or isoproterenol. In the presence of 1-34 bPTH [10^{-7} M], Ca^{++} uptake was significantly increased by 55% ($P < 0.001$) above control, while 3-34 bPTH [10^{-7} M] had no effect on uptake. Both 8br,cAMP [10^{-6} M] and dibut,cAMP [10^{-6} M] also significantly increased ($P < 0.001$) Ca^{++} uptake above control by 63 and 44%, respectively. Similarly, forskolin [10^{-5} M], the adenylate cyclase activator, increased Ca^{++} uptake by 41%. We next evaluated Ca^{++} efflux, and found that 1-34 bPTH [10^{-7} M], 1-84 bPTH [10^{-7} M], and forskolin [10^{-5} M] also increased Ca^{++} efflux by 50, 73, and 120%, respectively, above control. Since Ca^{++} transport was increased by either PTH, cAMP, or forskolin, we decided to determine if PTH action on Ca^{++} transport in synaptosomes was dependent on cAMP. This was investigated by measuring cAMP production during the conversion of ^{32}P -ATP to ^{32}P -cAMP in the presence of an ATP regenerating system (30 μg creatine phosphokinase, 10 mM creatine phosphate), and the cyclic nucleotide phosphodiesterase inhibitor (1 mM IBMX). Whereas forskolin [10^{-4} M] and NaF [100 mM] significantly increased ($P < 0.001$) adenylate cyclase activity in synaptosomes by eight- and fourfold, respectively, neither 1-34 bPTH nor 1-84 bPTH increased synaptosomal cyclase activity. However, in canine renal cortical plasma membranes (CRCPM), we observed significant increases in cAMP production with either forskolin, NaF, or PTH. Finally, to determine if synaptosomes contain an intact adenylate cyclase system, we measured cAMP production in the presence of the beta adrenergic agent, isoproterenol. Isoproterenol signifi-

cantly increased adenylate cyclase activity in both synaptosomes (90%) and CRCPM (50%).

These data suggest that although there is an intact adenylate cyclase system in rat brain synaptosomes, PTH-stimulated calcium transport in synaptosomes appears to be independent of this system.

Introduction

Previously, we investigated the effects of uremia on calcium transport in synaptosomes that were isolated from rat brain and found that both the Na-Ca exchanger and the ATP-dependent calcium pump were altered by uremia (1). The nature of the alteration was such that calcium accumulation was increased in the uremic vesicles by both these transport mechanisms. We further observed that when parathyroidectomy was performed in rats before they were made acutely uremic, the transport alterations in uremia were no longer seen (2). Additionally, the administration of PTH to parathyroidectomized rats that were then made uremic, resulted in a return of the calcium transport abnormalities. The magnitude of the alteration in this group of rats was similar to that which was observed in the nonparathyroidectomized uremic group. In non-uremic rats, the absence or presence of PTH also affected calcium transport in a manner similar to that which we observed in uremia: parathyroidectomy and PTH administration decreased and increased calcium transport, respectively (3). Based on these observations we decided to investigate the mechanism of action of PTH on calcium transport in rat brain synaptosomes to determine if its action was cAMP-mediated, as has been classically described in tissues such as bone (4) and kidney (5-7).

Methods

Isolation of synaptosomes

Synaptosomes were isolated from 200-g male Sprague-Dawley rats as we previously described (8). In brief, the rats were decapitated and their cerebral cortex removed and immediately placed in 10 ml of ice-cold isolation media containing 320 mM sucrose, 0.2 mM K-EDTA, 5 mM Tris-HCl (pH 7.4) at 0-4°C. The brains were chopped finely with scissors, washed with the isolation media, and homogenized in a glass Dounce homogenizer (clearance 1 mm). The crude synaptosomal-mitochondrial pellet was obtained after several centrifugations at 1,300 g for 3 min and a final spin at 18,000 g for 10 min. The purified synaptosomal preparation was then obtained by differential centrifugation on a discontinuous Ficoll gradient for 60 min (8). The synaptosomal protein was diluted in isolation media to a final concentration of 8-10 mg/ml and used for transport studies.

Calcium transport assay

Uptake studies. Calcium uptake studies were carried out by the Na-Ca exchange mechanism as previously described (1). An 0.5-ml aliquot of

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synaptosomal protein (~ 5 mg protein) was brought up to 2 ml in a preequilibration media (140 mM NaCl, 1 mM MgSO₄, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4) and allowed to incubate for 10 min at 37°C. The suspension was then spun at 20,000 *g* for 5 min and the resulting pellet was resuspended in the same media and spun at the same speed for 5 min. At the end of the second spin, the final pellet was resuspended in 400 μ l of the preequilibration media and kept on ice (0–4°C) until transport studies were commenced.

Uptake was initiated by adding 5 μ l of synaptosomal suspension (~ 50 μ g protein) to 95 μ l of an external media consisting of 140 mM KCl, 1 mM MgSO₄, 10 mM glucose, 10 μ M CaCl₂, 0.1 μ Ci ⁴⁵Ca⁺⁺ (40,000 cpm/20 μ l), 5 mM Hepes-Tris, pH 7.4 at 25°C. Depending on the compound being studied, either 100 ng/ml bovine parathyroid extract, 10⁻⁷ M 1-34 bPTH, 10⁻⁷ M 1-84 bPTH, 10⁻⁶ M 8bromocAMP, 10⁻⁶ M dibut-cAMP, or 10⁻⁵ M forskolin was added to the external media before transport study was begun. After the desired period of incubation, uptake was terminated by adding to the uptake media 2 ml of ice-cold 150 mM KCl solution (stop solution). The mixture was immediately vacuum filtered through a 0.45- μ m pore size cellulose acetate filter and washed twice with 2 ml of the cold stop solution as previously described (1). The filters were dissolved in phase combine scintillant and counted by a Packard counter (model 2000CA; Packard Instrument Co., Inc., Downers Grove, IL). Each time point was done in triplicate and the value obtained at time zero, which we attributed to binding of calcium to protein and filter, was subtracted from each of the points of observation (1, 8). As we described previously, nonspecific binding to the filter and synaptosomal protein was < 0.05% (1).

Efflux studies. For calcium efflux studies the vesicles are loaded by the Na-Ca exchanger for 10 min as discussed above. At the end of this period, the loaded vesicles are spun at 20,000 *g* for 10 min and resuspended to a protein concentration of 10 mg/ml in the external media (140 mM KCl, 1 mM MgSO₄, 10 mM glucose, 10 μ M CaCl₂, 0.1 μ Ci ⁴⁵Ca⁺⁺ 40,000 cpm/20 μ l, 5 mM Hepes-Tris, pH 7.4) and kept on ice until efflux studies were commenced. Efflux was promptly started by diluting 5 μ l of the calcium-loaded vesicles in 95 μ l of a calcium-free efflux media consisting of 140 mM NaCl, 1 mM MgSO₄, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4 at 25°C with appropriate additions of either 10⁻⁷ M 1-34 bPTH, 10⁻⁷ M 1-84 bPTH, 10⁻⁶ M 8bromocAMP, 10⁻⁶ M dibut-cAMP, or 10⁻⁵ M forskolin. At the appropriate period of efflux the reaction was terminated by adding 2 ml of ice-cold stop solution to 100 μ l of the transport mixture (9). The mixture was then filtered as above, washed twice with the stop solution, and counted. The initial time point was obtained at the instant before starting the efflux studies and taken as the maximum value of calcium where comparison was made over time.

Purification of canine renal cortical plasma membranes

Highly purified renal cortical plasma membranes were isolated from freshly removed canine kidney at 0–4°C. Cortical tissue was dissected free from medullary tissues, cut into small pieces with scissors, and washed in 0.9% NaCl, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5. The medium was decanted and the tissue was disrupted in 3 vol (relative to wet weight) ice-cold 0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5 (SET buffer),¹ using two to three short bursts (< 5 s) of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Tissue was further homogenized using 10 strokes of a motor-driven loose Teflon pestle. After centrifugation at 1,475 *g* for 10 min, the pellets were washed once with SET buffer then resuspended in 1 vol (relative to initial wet weight) 2 M sucrose, 1 mM EDTA, and 5 mM Tris-HCl, pH 7.5. The suspension was spun at 13,300 *g* for 10 min and the supernatant was decanted and diluted eightfold with 1 mM EDTA, 5

mM Tris-HCl, pH 7.5. This suspension was centrifuged at 20,000 *g* for 15 min and the white upper layer of the pellet was gently removed, resuspended in SET buffer, and respun. This process of centrifugation and resuspension was repeated three times to yield a homogeneous white fluffy pellet (10).

Guanyl nucleotide-amplified adenylate cyclase assay

Adenylate cyclase activity was measured by the conversion of [alpha-³²P]ATP to [³²P]cAMP. Standard incubations were carried out in a final volume of 0.1 ml containing 25 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.1% BSA, 0.1 mM [alpha-³²P]ATP (100–300 cpm/pmol), an ATP regenerating system consisting of 30 μ g creatine phosphokinase and 10 mM creatine phosphate, and 1 mM 1-methyl,3-isobutylxanthine, a potent inhibitor of cyclic nucleotide phosphodiesterase (11). GTP at final concentrations of 100 μ M was sometimes added to augment the sensitivity of adenylate cyclase to PTH (10). Appropriate concentrations of either 1-34 bPTH, 1-84 bPTH, forskolin, NaF, or isoproterenol were also added depending on experimental protocol. Incubation tubes were made of borosilicate glass and were pretreated with dichlorodimethylsilane (Aldrich Chemical Co., Inc., Milwaukee, WI) to reduce adsorption of PTH. Incubations were started by adding either canine renal cortical plasma membranes (CRCPM) or synaptosomes in SET buffer or buffer alone for assay blank. After 30 min of incubation at 30°C, enzyme activity was terminated by the addition of 0.1 ml of a solution containing 10 mM unlabeled ATP, 2% SDS (vol/vol), 50 mM Tris-HCl at pH 7.5, 1 mM [³H]cAMP (30,000 cpm). Assay tubes were immediately placed in a boiling water bath for 3 min and [³²P]cAMP was isolated by chromatographic procedure (10, 12). For studies with hypotonically lysed synaptosomal vesicles, lysis was achieved with 20 mM Tris-HCl as previously described (9).

Protein assay

Protein determination on the membrane preparations were determined by the methods of Lowry and associates using BSA as the reference standard (13).

Materials

Highly purified bovine PTH tetratriacontapeptide [1-34 bPTH, 1-84 bPTH, 3-34 bPTH], [alpha-³²P]ATP, [³H]cAMP, and Gpp(NH)p were all generous gifts from Dr. Claude Arnaud (Endocrine Division, VA Medical Center, San Francisco, CA). ATP, cAMP, forskolin, 1-methyl,3-isobutylxanthine, creatine phosphate, creatine phosphokinase, forskolin, and sodium fluoride were obtained from Sigma Chemical Co. (St. Louis, MO). ⁴⁵Ca⁺⁺ was obtained from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and obtained from Sigma Chemical Co.

Results

We first performed calcium uptake by the Na-Ca exchanger in synaptosomes as described in Methods and observations were made between 3 and 60 s. As shown in Fig. 1, with either 100 ng/ml of parathyroid extract or 10⁻⁷ M 1-34 bPTH, uptake was significantly increased (*P* < 0.001) from 0.31±0.01 to 0.44±0.01 and 0.48±0.01 nmol/mg protein, respectively, at 5 s. This PTH stimulation resulted in an increase in uptake of 42% with the extract and 55% with 1-34 bPTH. In the presence of PTH, uptake was significantly increased only up to 15 s; thereafter no difference was observed between PTH and the control group (Fig. 1). This suggests that the ability of PTH to stimulate calcium uptake in synaptosomes is transient and may be related to a finite amount of available substrate (ATP) within the vesicles. However, as we previously described, synaptosomes contain ~ 0.35 mM ATP, which is adequate to drive the system for more than 15 s, as will be shown later (8).

1. *Abbreviations used in this paper:* CRCPM, canine renal cortical plasma membrane; DAG, diacylglycerol; IP3, inositol trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; SET buffer, 0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5.

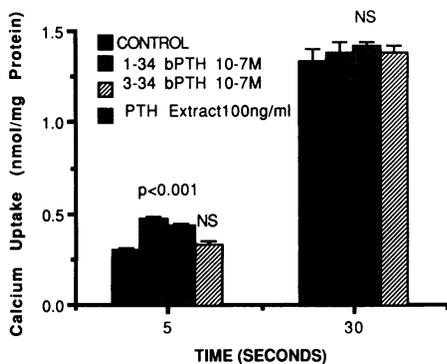


Figure 1. This graph shows the effects of three different PTH molecules on calcium uptake in synaptosomes. Uptake in the presence of either 100 ng/ml of parathyroid extract or 10^{-7} M 1-34 bPTH significantly increased calcium uptake above control at 5 s ($P < 0.001$). 10^{-7} M 3-34 bPTH had no stimulatory effects on uptake. At 30 s uptake of all three groups was not different from control. Data are expressed as mean \pm SE of eight experiments.

As opposed to 1-34 bPTH and the extract, 3-34 bPTH did not change calcium uptake from control values during the period of observation. To determine if calcium uptake in synaptosomes was stimulated by cAMP as was observed with PTH, we next performed uptake studies in the presence of 10^{-6} M of either 8-bromo-cAMP or dibut-cAMP for up to 5 min (Fig. 2). Between 3 and 30 s, both cAMP molecules significantly increased ($P < 0.005$) calcium uptake in a manner that was similar to PTH. At 5 s 8-bromo-cAMP significantly increased uptake by 63% from 0.32 ± 0.02 to 0.52 ± 0.03 nmol/mg protein, and dibuteryl-cAMP increased uptake by 44% above control. However, by 5 min, calcium uptake was no longer stimulated by cAMP and the values in all three groups were not significantly different from each other (Fig. 2). Based on these data, it appears that PTH may stimulate calcium transport in synaptosomes by a cAMP-mediated action. Thus, to investigate this possibility we first decided to study the action of forskolin on calcium transport, in order to determine if direct stimulation of the adenylate cyclase catalytic subunit by

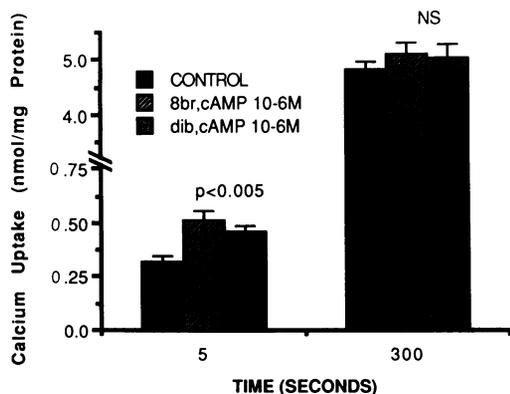


Figure 2. This graph shows the effects of 8-bromo-cAMP and dibut-cAMP on calcium uptake. In the presence of cAMP, uptake is significantly greater than in control vesicles at 5 s. No difference in uptake was observed between the groups at 5 min, by which time maximum uptake was achieved. Data are expressed as the mean \pm SE of 10 experiments.

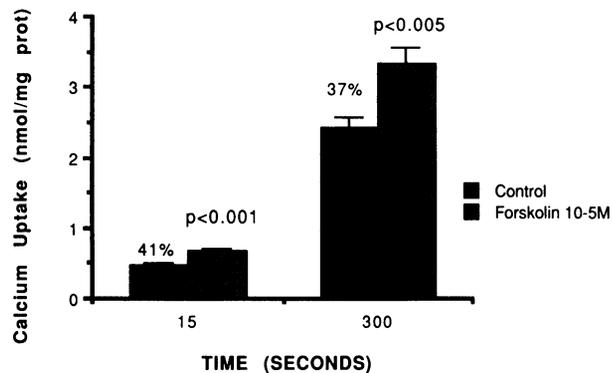


Figure 3. 10^{-5} M forskolin, activator of the catalytic subunit of adenylate cyclase, increased calcium uptake by 41 and 37% at 15 s and 5 min, respectively. Data are expressed as the mean \pm SE of nine experiments.

forskolin (14) would increase synaptosomal calcium transport, as was observed with PTH.

Calcium uptake was assessed by adding 10^{-5} M forskolin to the external media and following transport over time (3 s to 10 min). Throughout the period of observation (Fig. 3), calcium uptake in the presence of forskolin was significantly increased ($P < 0.005$) above control by $\sim 40\%$. This prolonged stimulation of calcium uptake by forskolin was distinctly different from the transient increases observed with either PTH or cAMP. Since forskolin activates the cyclase system by binding to the catalytic subunit, it is evident that the native intrasynaptosomal ATP is sufficient to produce a sustained uptake by PTH.

We next decided to determine if calcium efflux from synaptosomes was also affected by PTH. As shown in Fig. 4, efflux in the presence of 10^{-7} M 1-34 bPTH was significantly increased ($P < 0.005$) above control by 29–50% between 3 and 10 s. Similarly, 10^{-7} M 1-84 bPTH also significantly increased calcium efflux above control by 32–73% at the same time interval. With 10^{-5} M forskolin, efflux was increased above control by 42–120%, again suggesting the possible dependence of PTH stimulation of calcium transport on cAMP. To ensure that these differences in calcium transport were due to active

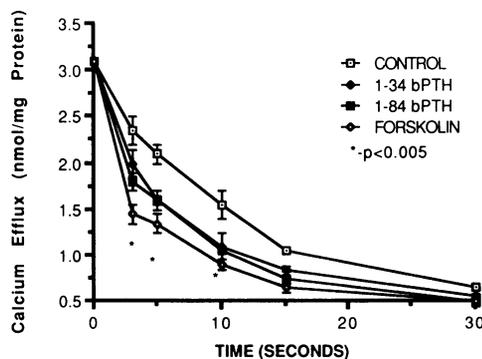


Figure 4. This graph shows calcium efflux from synaptosomes in the presence of 10^{-7} M of either 1-34 bPTH or 1-84 bPTH. In the presence of PTH calcium efflux is significantly greater ($P < 0.005$) than in control vesicles between 3 and 10 s. Data are expressed as mean \pm SE of eight experiments.

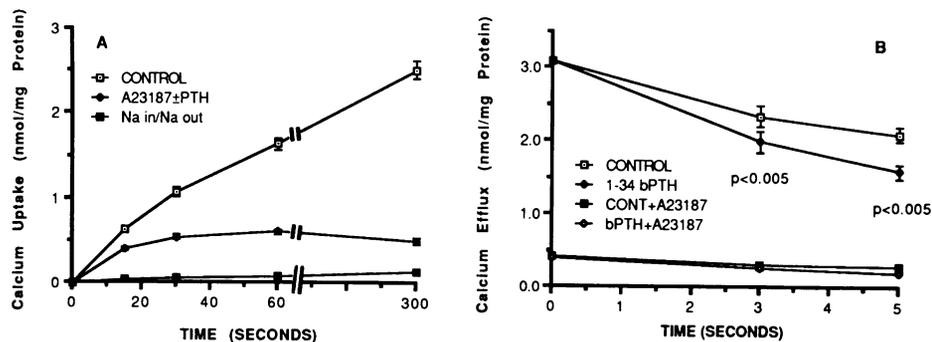


Figure 5. The graph in *A* shows the effect of the calcium ionophore A23187 and the absence of a sodium gradient (Na in/Na out) on calcium uptake in synaptosomes. In the absence of a gradient, calcium uptake was only ~ 5% of control values. A23187 also prevented the vesicles from actively taking up calcium. In *B*, A23187 preincubated in loading media prevented the vesicles uptaking calcium and thus equilibrated calcium concentration inside and outside of the vesicles. In the absence of A23187 significant difference in efflux was observed between control and PTH-treated vesicles. Data are expressed as mean±SE of eight experiments.

uptake and efflux of calcium by the sodium/calcium exchanger and not nonspecific binding, we simultaneously performed transport studies in the presence of the calcium ionophore A23187, and also measured uptake in the absence of a sodium gradient (Fig. 5). As demonstrated in Fig. 5 *A*, in the absence of a sodium gradient (Na in/Na out), no significant uptake was observed over 5 min of observation. Similarly, the ionophore A23187 equilibrated the calcium concentration between the inside and outside of the vesicles, thus preventing the active uptake that is observed in the presence of a sodium gradient and the absence of the ionophore (Fig. 5 *A*). In efflux studies, A23187 also eliminated the observed differences in transport between control vesicles and those treated with PTH (Fig. 5 *B*). Both these studies and the subtraction of time zero counts from ensuing points (see Methods) suggest that nonspecific binding of calcium to synaptosomes does not influence the results of these studies. These observations are similar to our previously published findings (1). In considering the entire data thus far, we felt that the preponderance of the evidence suggested that PTH-dependent calcium transport in synaptosomes was dependent on cAMP. To determine if this was the case, we then measured cAMP production rates in synaptosomes in the presence of either PTH or the membrane adenylate cyclase activators forskolin and sodium fluoride (NaF). These three compounds were used in order to systematically analyze the three distinct subunits of the hormone-responsive adenylate cyclase system that are responsible for the generation of cAMP (14).

As explained in Methods, cAMP production was evaluated during the conversion of [α - 32 P]ATP to [α - 32 P]cAMP in the presence of both an ATP regenerating system and an inhibitor of cyclic nucleotide phosphodiesterase. The purpose of these additions was to provide enough substrate to maintain adequate ATP concentration during the production of cAMP, and also to prevent the breakdown of cAMP once it was formed. Studies were performed simultaneously in CRCPM and in synaptosomes. The CRCPMs were used as control for the studies in synaptosomes. Table I shows that the addition of either 30, 300, or 100,000 ng/ml of 1-34 bPTH resulted in ~ 3-, 4.5-, and 5-fold increase in adenylate cyclase activity in CRCPM, respectively. At the same time no increase in cyclase activity was observed in synaptosomes with the same concentrations of 1-34 bPTH. Similarly, with either 10 or 500 ng/ml

of 1-84 bPTH, adenylate cyclase activity increased from 250 ± 15 to 519 ± 22 or to $1,036 \pm 45$ pmol cAMP/mg protein in CRCPM, an increase of two- and fourfold, respectively. In synaptosomes, cyclase activity was also unaffected by 1-84 bPTH ($1,300 \pm 98$ to $1,357 \pm 34$ or $1,290 \pm 75$ pmol cAMP/mg protein). To determine if either the catalytic or the guanine nucleotide regulatory subunits were operative in synaptosomes, we then carried out experiments in the presence of 0.1 mM forskolin, activator of the catalytic subunit of the adenylate cyclase system (14). In CRCPM (Table I), adenylate cyclase activity increased from 239 ± 34 to $3,330 \pm 145$ pmol cAMP/mg protein, and in synaptosomes from 911 ± 51 to $7,466 \pm 420$ pmol cAMP/mg protein. Similarly, 100 mM NaF (activates adenylate cyclase through the stimulatory guanine nucleotide regulatory subunit) increased cyclase activity from 239 ± 34 to $6,669 \pm 310$ in CRCPM and from 911 ± 51 to $3,562 \pm 248$ in synaptosomes. These results suggest that both the catalytic and regulatory subunits of the adenylate cyclase system are intact

Table I. Adenylate Cyclase Activity Measured as a Function of Increasing Concentration of Known Cyclase Activators

Agents	Concentration	Adenylate cyclase activity	
		CRCPM	Synaptosomes
<i>pmol cAMP/mg prot. 30 min</i>			
No. of experiments		5	5
1-34 bPTH (ng/ml)	0	278±11	911±51
	30	763±25 (2.7)	—
	300	1,230±36 (4.4)	953±57
	100,000	1,411±40 (5.1)	922±44
1-84 bPTH (ng/ml)	0	250±15	1,300±98
	10	519±22 (2.1)	1,357±34
	500	1,036±45 (4.1)	1,290±75
Forskolin (mM)	0	239±34	911±51
	0.1	3,330±145 (14)	7,466±420 (8.2)
NaF (mM)	0	239±34	911±51
	100	6,669±310 (28)	3,562±248 (4)

Activity was measured in the presence of 1 mM IBMX, 10 mM creatine phosphate and 30 μ g creatine phosphokinase. Parentheses indicate the fold increase in cyclase activity above control. Data are expressed as mean±SE; each time point was done in triplicate.

and functional in synaptosomes, and that the mechanism of action of PTH on calcium transport in synaptosomes may be independent of cAMP.

To determine if the tightness of the synaptosomal membrane prevented the externally added ^{32}P -ATP from entering the vesicle, resulting in the labeled ATP being unavailable for conversion to ^{32}P -cAMP, we next measured cAMP production in both intact and lysed synaptosomes (Fig. 6). No difference in cAMP production was observed between the intact and the hypotonically lysed synaptosomes. Thus, it does not appear that the inability of synaptosomes to produce cAMP by PTH is due to the unavailability of the added ATP. In experiments that were simultaneously performed in CRCPM, increasing concentration of PTH resulted in increased cAMP production (Fig. 6).

To investigate if PTH effect on calcium transport was independent of cAMP, it was necessary for us to determine if an intact adenylate cyclase system exists in synaptosomes. To achieve this, we evaluated adenylate cyclase activity in synaptosomes in the presence of isoproterenol, a beta adrenergic agonist that is a well-known adenylate cyclase activator (14). Table II shows that 10 mM isoproterenol resulted in 90% increase in cyclase activity in synaptosomes and 50% increase in CRCPM, and suggests that there is an intact adenylate cyclase system in synaptosomes that is at least active as in CRCPM. Therefore, the inability of PTH to stimulate cAMP production in synaptosomes does not appear to be due to the absence of an intact adenylate cyclase system, but to an inability of PTH to activate the cyclase system in synaptosomes.

Discussion

In the studies described above, it is shown that PTH increases both the uptake and the efflux of calcium from rat brain synaptosomes in a manner similar to either cAMP or forskolin. Based on these findings, it was initially felt that the mechanism

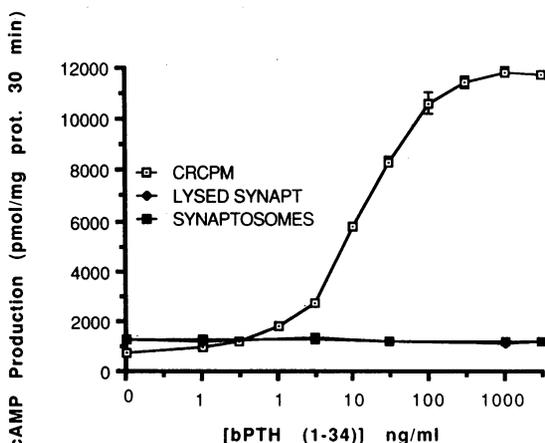


Figure 6. This graph shows the effects of PTH on adenylate cyclase activity in CRCPM, intact synaptosomes, and lysed synaptosomal plasma membranes (LYSED SYNAPT). In CRCPM increased concentration of 1-34 PTH results in increased production of cAMP, however, cAMP production was not increased in either of the synaptosomal preparations. The experiment was carried out in the presence of GTP, IBMX, creatine phosphate, and creatine phosphokinase. Data are expressed as mean \pm SE.

Table II. Ability of 1-34 bPTH and the Beta Adrenergic Agonist Isoproterenol to Activate Adenylate Cyclase Activity in Synaptosomes and CRCPM

Agents	Concentration	Adenylate cyclase activity	
		CRCPM	Synaptosomes
<i>pmol cAMP/mg prot. 30 min</i>			
1-34 bPTH (ng/ml)	0	242 \pm 8	1,080 \pm 41
	30	3,357 \pm 41 (14)	1,198 \pm 37
	1,000	4,363 \pm 57 (18)	1,148 \pm 16
Isoproterenol (μM)	0	253 \pm 14	1,116 \pm 21
	100	390 \pm 21 (1.5)	1,307 \pm 7 (1.2)
	10,000	367 \pm 7 (1.5)	2,099 \pm 114 (1.9)

Activity was measured in the presence of GTP, 1 mM IBMX, 10 mM creatine phosphate, and 30 μg creatine phosphokinase. Parentheses indicate the fold increase in cyclase activity due to the agents. Data are expressed as mean \pm SE.

of action of PTH on calcium transport in synaptosomes was cAMP dependent. It was also observed that parathyroid extract and 1-34 bPTH increased synaptosomal calcium transport, while 3-34 bPTH had no such effect on uptake (Fig. 1). This suggested to us that the amino acids in positions 1 and 2 of the PTH molecule are necessary for PTH to stimulate calcium transport in synaptosomes. As a result of these observations, we used 1-34 bPTH and 1-84 bPTH in all subsequent studies, and the results indicate that both PTH molecules produced similar responses in calcium transport and cAMP generation in synaptosomes.

As mentioned above, our initial observations that PTH stimulated calcium transport in synaptosomes was similar to that observed with cAMP and forskolin (Figs. 1-4) lead us to believe that this mechanism of action of PTH was cAMP dependent. However, the inability of PTH to generate cAMP in synaptosomes suggested to us that the relationship between PTH and synaptosomal calcium transport was more complicated than we initially thought. This complexity became more intriguing when PTH increased cyclase activity in CRCPM by fivefold in simultaneously run experiments (Table I). Our aim, then, was to determine if there was an intact adenylate cyclase system in synaptosomes, and whether its function could be demonstrated by other known activators of the adenylate cyclase system. Our first task was to measure cAMP production in synaptosomes and CRCPM in the presence of either forskolin or NaF and to compare the results in these two membrane preparations. In both preparations, forskolin as well as NaF increased cyclase activity many fold (Table I), suggesting that synaptosomes contain both a catalytic and the guanine nucleotide regulatory subunits that are similar to that in CRCPM.

We next measured cAMP production in both membrane preparations in the presence of isoproterenol, a beta adrenergic agonist, which produces many of its functions by first binding to surface receptors, causing an increase in cAMP production and activation of a cAMP-dependent protein kinase (15). As shown previously (Table II), 1-34 bPTH increased adenylate cyclase activity 18-fold in CRCPM, while it had no effect on the cyclase activity in synaptosomes. However, with isoproterenol, cyclase activity was increased by 50% in CRCPM and 90% in synaptosomes. This suggested that like CRCPM, synaptosomes contain an intact adenylate cyclase system that can

be stimulated by cyclase activators other than PTH. Thus, it appears that the PTH-stimulated calcium transport observed in synaptosomes is independent of cAMP.

Under other conditions where a hormone-responsive adenylate cyclase system exists, the role of the hormone in eliciting cAMP responses can be demonstrated by measuring cAMP production in the presence of the hormone (14, 16). Examples of this relationship include: inhibition of platelet aggregation by prostaglandins (17), stimulation of lipolysis in adipocytes, stimulation of steroidogenesis in rat adrenal cells, relaxation of peripheral smooth muscle, and enhancement of secretion from blowfly salivary glands (14). However, in our studies (Table I), both 1-34 and 1-84 bPTH failed to produce cAMP in synaptosomes, despite the presence of an intact adenylate cyclase system (Table II). This again suggests that PTH action on calcium transport in brain synaptosomes may not require the presence of cAMP.

As with our observations in synaptosomes, studies in other tissues have shown that PTH can produce cellular changes that are independent of the adenylate cyclase system. In a recent study it was shown by Hruska and associates that PTH increased intracellular calcium in proximal tubular cells of the mammalian kidney independently of the cyclic nucleotides (18). Similarly, in the opossum kidney cell line, which exhibits proximal tubular characteristics, it was also shown that PTH increased cytoplasmic calcium and produced a dose-dependent stimulation of inositol triphosphate (IP₃) and diacylglycerol (DAG) which was not mimicked by cAMP (19). In the isolated papillary muscle from rat heart, PTH action on papillary muscle contraction was also shown to be independent of cAMP (20). In this study it was shown that the inotropic action of PTH on papillary muscle was mediated by the release of endogenous norepinephrine from the myocardium, and that both the release of norepinephrine and muscle contraction were independent of adenylate cyclase activation (20). Thus, it is evident from these studies that the mechanism of action of PTH on cellular functions is quite variable and does not necessarily involve the adenylate cyclase system. The data from our studies support these findings and our contention that PTH action on calcium transport in rat brain synaptosomes may be independent of cAMP.

A recent study has shown that inositol 1,3,4,5-tetrakisphosphate (IP₄) activates sea urchin eggs by a mechanism that was dependent on external calcium (21). Similarly, hepatocytes whose functions are coupled to the inositides stimulated by an agonist such as vasopressin, produced a prolonged increase in intracellular calcium concentration that is dependent on extracellular calcium (22). IP₄ has been shown to rise rapidly in rat cerebral cortical slices after muscarinic receptor stimulation (23), and to decline just as rapidly by inhibition of the receptor (23, 24). Thus, it appears that after the agonist binds to surface receptors, phosphoinositol, a membrane-bound lipid, is converted to DAG and IP₃, which is then phosphorylated to IP₄. IP₄ by itself and/or with IP₃ or DAG changes the membrane permeability of the cell to calcium so that there is an influx of calcium from the extracellular space to the cytosol (21). The increased intracellular calcium that is produced as a result of this mechanism is different from that which is observed after the release of microsomal calcium by IP₃ in tissues such as liver, muscle, bag cell neurons, and kidney (19, 25, 26). This regulation of intracellular calcium from extracellular sources by an agonist is an important one and

might be a possible mechanism of action of PTH in synaptosomes.

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