Chronic Parathyroid Hormone Excess In Vivo Increases Resting Levels of Cytosolic Calcium in Brain Synaptosomes: Studies in the Presence and Absence of Chronic Renal Failure

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ABSTRACT
It has been suggested that excess parathyroid hormone (PTH) in chronic renal failure (CRF) or chronic administration of PTH to normal rats caused derangements in norepinephrine and phospholipid metabolism of brain synaptosomes, because of an increase in their resting levels of cytosolic calcium which may induce a decrease in synaptosomal content of ATP. In the study presented here, the resting levels of cytosolic calcium in brain synaptosomes were measured in six groups of rats including: (1) normal rats; (2) rats with CRF of 21-days duration; (3) hyperparathyroidemic parathyroidectomized rats with CRF of 21-days duration; (4) rats with CRF of 21-days duration treated with verapamil from day 1 of CRF; (5) normal rats treated with verapamil for 21 days; and (6) normal rats treated with PTH for 21 days. Resting levels of cytosolic calcium in brain synaptosomes of CRF rats (437 ± 18.0 nM) and normal rats treated with PTH (428 ± 5.6 nM) were significantly (P < 0.01) higher than those of normal rats (345 ± 9.0 nM), normal rats treated with verapamil (354 ± 8.7 nM), CRF rats treated with verapamil (361 ± 12.9 nM), or CRF-parathyroidectomized rats (363 ± 8.2 nM). There were no significant differences between the values of the last three groups of rats. The ATP content of brain synaptosomes of CRF rats (2.95 ± 0.23 nmol/mg of protein) and normal rats treated with PTH (3.06 ± 0.13 nmol/mg of protein) were significantly (P < 0.01) lower than that in normal rats (4.49 ± 0.30 nmol/mg of protein), normal rats treated with verapamil (4.60 ± 0.30 nmol/mg of protein), CRF rats treated with verapamil (4.05 ± 0.25 nmol/mg of protein), or CRF-parathyroidectomized rats (4.03 ± 0.22 nmol/mg of protein). There were no significant differences between the values of the last three groups of animals. The data demonstrate that chronic excess of PTH in the presence or absence of CRF is associated with significant elevations in the resting levels of cytosolic calcium of brain synaptosomes and significant reduction of their ATP content.

Key Words: Brain synaptosomes, cytosolic calcium, hyperparathyroidism, chronic renal failure, verapamil

Several lines of evidence indicate that acute exposure to parathyroid hormone (PTH) enhances entry of calcium into many cells (1-4) including brain synaptosomes (5). Similarly, a significant increase in synaptosomal content of total calcium has been noted in experimental models with chronic exposure to excess PTH, such as rats with chronic renal failure (CRF) or after prolonged administration of PTH to rats with normal renal function (6). In these animals with chronic exposure to PTH, there were many abnormalities in norepinephrine and phospholipid metabolism of brain synaptosomes (6,7). It was suggested that the increase in synaptosomal total calcium may reflect a rise in the levels of resting cytosolic calcium of these structures, and this latter change is responsible for the derangement in synaptosomal function and metabolism (6,7). A number of in vivo studies with chronic excess of PTH have also shown that the PTH-induced increase in calcium content of many organs such as the heart (8), skeletal muscle (9), and pancreas (10) caused a decrease in ATP production and hence reduced ATP content of these organs. It is, therefore, possible that a rise in the resting levels of cytosolic calcium in brain synaptosomes in animals with chronic excess of PTH is also associated with a fall in their ATP content. Such a change in ATP could provide another
pathogenetic pathway through which chronic exposure to PTH may adversely affect the functional integrity of brain synaptosomes.

Data documenting that chronic exposure to excess PTH causes a rise in the resting levels of cytosolic calcium of brain synaptosomes and/or a fall in their ATP content are lacking. The demonstration of such effects is critical for the understanding of the mechanisms underlying the derangements in brain function in CRF because the latter is associated with sustained secondary hyperparathyroidism (11,12). The study presented here examined the effect of the chronic exposure to excess PTH in the presence or absence of CRF on the resting levels of cytosolic calcium and on ATP content of brain synaptosomes.

METHODS

Male Sprague-Dawley rats weighing 280 to 370 g were studied. They were fed rat chow diet (Wayne Research Animal Diets, Chicago, IL) throughout the study and allowed to drink ad libitum. The diet contained 1.4% calcium and 0.97% phosphorus. Six groups of animals were studied including: (1) normal rats; (2) rats with CRF of 21-days duration; (3) normocalcemic parathyroidectomized (PTX) rats with CRF of 21-days duration; (4) rats with CRF of 21-days duration treated with verapamil from day 1 of CRF; (5) normal rats treated with verapamil for 21 days; and (6) normal rats treated with PTH for 21 days. The rats were assigned randomly to these various groups.

Parathyroidectomy was done by electrocautery, and the success of the procedure was ascertained by a decrease in plasma levels of calcium of at least 2 mg/dL. The PTX rats were allowed to drink freely water containing 50 g/L of calcium gluconate. This procedure is adequate to normalize plasma calcium in PTX rats. Rats that did not display a fall in plasma calcium of 2 mg/dL or more after PTX and PTX rats that did not attain normocalcemia with drinking calcium-containing water were discarded and not used in the study. Seven days after PTX, the animals underwent right partial nephrectomy through a flank incision; a week later, a left nephrectomy was performed. This nephrectomy procedure was also performed in rats with intact parathyroid glands. 1-84 PTH (Sigma Chemical Co., St. Louis, MO) was dissolved in normal saline and given intraperitoneally in a dose of 75 μg twice daily. Verapamil (Isotopen, Knoll, AG, Ludwigshafen, Federal Republic of Germany) was injected subcutaneously in a dose of 0.1 μg/kg body wt twice daily. The normal rats received intraperitoneal injections of vehicle only.

On the day of the study, the rats were sacrificed by decapitation. Blood was collected in heparinized tubes for the measurements of creatinine, calcium, and phosphorus. The forebrains of the animals were immediately removed, placed on ice-cold (4°C) isolation media (320 mM sucrose, 0.2 mM K-EDTA, 5 mM Tris-HCl; pH 7.4), and chopped into small pieces with scissors. The chopped tissues were washed three times with isolation media. Synaptosomes were prepared according to the method of Booth and Clark (13). The details of the procedure for isolation of synaptosomes in our laboratory, as well as the various approaches to evaluate the intactness of the synaptosomes, their viability, and the degree of contamination with mitochondria and endoplasmic reticulum, have been previously reported from our laboratory (6,7). Each forebrain yielded synaptosomes containing 7 to 10 mg of protein which was diluted in 1.5 to 2.0 mL of isolation medium, and this preparation was used for the measurement of cytosolic calcium.

Cytosolic calcium of synaptosomes was measured with Fura2-AM (Sigma Chemical Co.) by a modification of the methods described by Komulainen and Bondy (14) and Verhage et al. (15). Samples of 250 μL of the synaptosomal preparation (1.5 to 1.8 mg of protein) were mixed with 5 mL of artificial cerebrospinal fluid (CSF) containing in mM: NaCl, 132; KCl, 3; MgSO4, 1; NaH2PO4, 1.2; d-glucose, 10; HEPES, 10; and CaCl2, 0.02. pH was adjusted to 7.4 with Tris buffer. The mixture was spun at 9,500 × g for 5 min with a Sorval RC-5 refrigerated centrifuge (4°C) (DuPont Co., Instrument Division, Newton, CT). The pellet was mixed with 0.5 mL of CSF fluid containing 4 μM Fura2-AM and was incubated at 37°C in a shaker water bath for 30 min. At the end of the incubation, 10 mL of cold CSF fluid was added and the mixture was centrifuged again for 5 min. The final synaptosomal pellet was diluted with 1.0 mL of CSF and was kept on ice for 4 to 10 min until the measurement of cytosolic calcium was made with a Perkin-Elmer fluorescence spectrophotometer model LS 5B (Perkin-Elmer Instrument Division, Norwalk, CT) at excitation wavelengths of 340 nm and 380 nm and emission wavelengths of 510 nm with a slit of 10 and 20 mm, respectively. Before each measurement, 100 μL of the synaptosomal preparation was centrifuged for 30 s with a Beckman microfuge (Beckman Instrument, Inc., Palo Alto, CA) and the pellet was directly added to the spectrophotometer cuvette containing 2 mL of CSF solution with 1 mM CaCl2 at a temperature of 37°C. After 2 to 3 min of temperature (37°C), equilibration measurements of cytosolic calcium was made. Maximal fluorescence (Fmax) and minimal fluorescence (Fmin) were estimated as previously reported (16,17). The cells were lysed with Triton (0.05%) to obtain Fmax. Next, 10 μL of 0.5 M EGTA in 3 M Tris base buffer were added to obtain Fmin. The pH was 8.5. To eliminate the effects of autofluorescence due to the cuvette, medium, or synapto-
somosomes, fluorescence was measured with empty cuvette after the addition of medium and after the addition of synaptosomes without Fura2. The unloaded synaptosomes produced very minimal and almost undetectable fluorescence. Correction for autofluorescence of the cuvette and medium was made by setting the fluorometer on autozero before each measurement. Calculation of cytosolic calcium was made by using the Grynkiewicz equation (17). The dissociation constant for Ca²⁺-Fura2 was assumed to be 225 nM.

The ATP content of synaptosomes was measured according to the method reported by Komulainen and Bondy (18). Fresh samples of 0.25 μL of synaptosomal preparation containing 1.5 to 3.0 mg of protein was sonicated for 15 s and then mixed with 0.52 mL of 0.6 M ice-cold perchloric acid. After 10 min of extraction on ice, 62.5 μL of 2 M K₂₂CO₃ was added and the solution was centrifuged at 13,500 × g for 5 min. The supernatant was removed, immediately frozen with liquid nitrogen, and stored at −70°C. On the day of the assay, the supernatant was thawed and diluted 10 times with 40 mM Tris buffer (pH 7.4). Samples of 50 μL were assayed for ATP by the firefly luminescence assay with a LAD 535 luminometer (Turner Design, Sunnyvale, CA). ATP standards were prepared with 40 mM Tris buffer (pH 7.4) and the same amounts of HC1O₄ and K₂₂CO₃ as that used in the synaptosomal preparation.

The measurements of calcium in plasma were made with a Perkin-Elmer atomic absorption spectrophotometer model 503, and those of creatinine and phosphorus were made with a Technicon automatic analyzer (Technicon Instrument Inc., Tarrytown, NY). PTH levels in serum were determined by an INS-PTH immunoassay kit (Nichols Institute Diagnostic, San Juan Capistrano, CA). This assay recognizes the amino-terminal fragment of PTH. The assay showed reproducible IC₅₀ for inhibition of [¹²⁵I]PTH binding of 21.4 ± 0.8 pg and less than 10% difference within one assay. The lower limit of detectability of PTH with this assay is 10 pg/mL. Statistical analysis used the unpaired t test.

RESULTS

Table 1 presents values of body weight and biochemical data of the various groups of rats studied. The CRF, CRF-PTX, CRF-verapamil, and PTH-treated rats weighed less than did the normal animals (P<0.01), but there were no significant differences between the weights of the animals in these four experimental groups. The nephrectomy procedure resulted in a significant (P<0.01) rise in the plasma concentrations of creatinine with the values in CRF, CRF-PTX, and CRF-verapamil rats being three to four times higher than that in normal rats. PTH treatment of normal rats did not cause a significant change in the levels of plasma creatinine. There were no significant differences in the plasma levels of calcium among the various groups of rats, but the plasma concentrations of organic phosphorus in CRF, CRF-verapamil, and PTH-treated rats were significantly (P<0.01) lower than that in the normal groups of rats. A low plasma phosphorus in animals with CRF comparable in degree to that in CRF rats of the study presented here has been observed (6,7). This may be due to the state of secondary hyperparathyroidism in the CRF rats which have moderate renal failure. The serum levels of PTH were significantly (P<0.01) higher in CRF rats and normal rats treated with PTH and significantly lower (P<0.01) in CRF-PTX animal than values in normal rats.

Figure 1 depicts fluorescence emission of Fura2 at 510 nm in response to 340- and 380-nm excitation along with Triton and EGTA calibration during the measurements of cytosolic calcium in brain synaptosomes from a normal rat. The changes of cytosolic calcium in response to 50 nM lonomycin and 50 mM KC1 are also shown. The resting level of cytosolic

TABLE 1. Body weight and plasma chemistry in the different groups of rats

<table>
<thead>
<tr>
<th></th>
<th>B Wt (g)</th>
<th>Plasma Creatinine (mg/dL)</th>
<th>Plasma Calcium (mg/dL)</th>
<th>Plasma Phosphorus (mg/dL)</th>
<th>Serum PTH (pg/mL)</th>
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<tbody>
<tr>
<td>Normal (N = 12)</td>
<td>302 ± 8.4</td>
<td>0.36 ± 0.01</td>
<td>10.2 ± 0.2</td>
<td>7.3 ± 0.2</td>
<td>45 ± 0.9</td>
</tr>
<tr>
<td>Normal-Verapamil (N = 12)</td>
<td>280 ± 8.9</td>
<td>0.34 ± 0.01</td>
<td>10.1 ± 0.4</td>
<td>6.7 ± 0.2</td>
<td>84 ± 6.3</td>
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<tr>
<td>CRF (N = 8)</td>
<td>254 ± 12.4</td>
<td>1.21 ± 0.05</td>
<td>10.0 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>96 ± 8.7</td>
</tr>
<tr>
<td>CRF-PTX (N = 8)</td>
<td>249 ± 11.8</td>
<td>1.03 ± 0.08</td>
<td>10.4 ± 0.3</td>
<td>7.6 ± 0.2</td>
<td>24 ± 0.4</td>
</tr>
<tr>
<td>PTH-treated (N = 8)</td>
<td>274 ± 4.0</td>
<td>0.35 ± 0.01</td>
<td>10.5 ± 0.2</td>
<td>6.4 ± 0.3</td>
<td>96 ± 8.7</td>
</tr>
<tr>
<td>CRF-Verapamil (N = 12)</td>
<td>263 ± 13.5</td>
<td>1.29 ± 0.09</td>
<td>10.4 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>96 ± 8.7</td>
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*Results are presented as means ± 1 SE; * P < 0.01 versus normal; † P < 0.05 versus normal-verapamil; ‡ P < 0.01 versus normal and CRF-PTX; § P < 0.01 versus normal.
Figure 1. Sample traces of fluorescence emission of Fura2 at 510 nm in response to 340- and 380-nm excitation along with effects of ionomycin, KCl, Triton, and EGTA calibration. These tracings were obtained in synaptosomal suspension from a normal rat. Continuous fluorescence depicts excitation of 340 nm, and interrupted fluorescence at the bottom of the figure shows excitation at 380 nm. The value of resting cytosolic calcium in this study was 315 nM and increased to 430 nM with ionomycin and to 618 nM with KCl.

calcium of brain synaptosomes in this study was 315 nM and increased to 436 nM with ionomycin and to 618 nM with KCl.

The levels of resting cytosolic calcium of brain synaptosomes in the various groups of animals are given in Figure 2. The values in normal rats was 345 ± 9.0 nM, and verapamil treatment of normal rats did not affect the resting levels of synaptosomal cytosolic calcium (354 ± 8.7 nM). CRF or PTH treatment of normal rats was associated with a significant (P<0.01) rise in resting levels of cytosolic calcium of brain synaptosomes with the values being 437 ± 18 nM and 428 ± 5.6 nM, respectively. This rise in cytosolic calcium of synaptosomes from CRF rats was corrected by verapamil treatment of these animals (361 ± 12.9 nM) or by PTX of these rats (363 ± 8.2 nM).

Figure 3 depicts the values of ATP content of brain synaptosomes in the various groups of animals. The values in CRF rats (2.95 ± 0.23 nmol/mg of protein) and PTH-treated animals (3.06 ± 0.13 nmol/mg of protein) were significantly (P<0.01) lower than those in normal rats (4.49 ± 0.30 nmol/mg of protein), normal rats treated with verapamil (4.6 ± 0.30 nmol/mg of protein), CRF-PTX rats (4.03 ± 0.22 nmol/mg of protein), and CRF-verapamil rats (4.05 ± 0.25 nmol/mg of protein). There were no significant differences in the values of synaptosomal ATP in the last four groups of animals.

**DISCUSSION**

Available data indicate that several aspects of the methodology used for the measurements of synaptosomal cytosolic calcium may affect its value. These factors include the technique of synaptosomal preparation (15), the type of fluorescent calcium indicator (14,15,19), and the calcium concentration of the loading media (15) and its temperature (15). It is not surprising, therefore, to find reports of normal cytosolic calcium in brain synaptosomes ranging from 200 to 400 nM (14,15,19). The values of 345 ± 9 nM obtained by us with the use of Fura2 is either not different or slightly higher than those measured by others with the same dye indicator (15,16,20). Despite the potential variation in the measurements of synaptosomal cytosolic calcium due to differences in methodology, changes in its value induced by experimental procedures should be considered real and
valid if the same method is used. This approach was used in our studies.

The results of the study presented here demonstrate that the resting levels of cytosolic calcium in brain synaptosomes of rats with CRF are elevated. Several lines of evidence indicate that this phenomenon is due to chronic excess of PTH. First, the blood levels of PTH in these animals were elevated. Second, PTX of CRF rats prevented the elevation in serum PTH levels and the rise in the resting levels of cytosolic calcium of synaptosomes. Third, elevation of the blood levels of PTH by the chronic administration of the hormone to rats with normal renal function caused a significant rise in synaptosomal levels of resting cytosolic calcium. Thus, it is apparent that chronic excess of PTH in the presence and absence of CRF is associated with a rise in the resting levels of cytosolic calcium of brain synaptosomes. A similar effect of chronic excess of PTH was found in the pancreatic islets of rats with CRF (21) or of those receiving chronic treatment with PTH (10). In these animals, the resting levels of cytosolic calcium of pancreatic islets were markedly elevated.

Data on the effect of acute exposure of synaptosomes from normal rats to PTH on their cytosolic calcium are not available. However, Fraser and Sarnecki (5) have shown that the acute exposure of brain synaptosomes to PTH was associated with increased influx of calcium into these structures. This finding is consistent with the notion that PTH may increase cytosolic calcium of synaptosomes.

Although both acute or chronic excess PTH may augment calcium entry into brain synaptosomes, one would expect that these structures would efficiently extrude this extra calcium and maintain their resting cytosolic calcium normal unless the balance between calcium entry into and its extrusion out of the synaptosomes is impaired. Calcium-activated ATPase (22,23) and Na+/Ca2+ exchanger (22–25) are the main processes involved in the extrusion of calcium out of the cells, and Na+/K+ ATPase activity, by regulating intracellular sodium, may be indirectly involved in the maintenance of intracellular calcium level. All of these pumps require ATP directly or indirectly for their function (23,26,27). It is, therefore, reasonable to suggest that the significant reduction in the ATP content of the synaptosomes of CRF rats and those treated with PTH may, at least partly, exert an adverse effect on the functional integrity of these pumps, resulting in a rise in the resting levels of cytosolic calcium. To accept this notion, one must provide direct evidence demonstrating that the activity of these pumps is indeed impaired in these experimental conditions. We have already reported that the Na+/K+ ATPase activity is reduced in synaptosomes from CRF rats and from animals treated with PTH (6). However, an adverse effect of CRF or PTH treatment on the function of synaptosomal calcium-activated ATPase and the Na+/Ca2+ exchanger awaits documentation. It is of interest that a comparable decrease in the ATP content of brain synaptosomes in another experimental model (phosphate depletion) was associated with a decrease in their Na+/K+ ATPase activity and V_max of Ca2+ ATPase and with a rise in cytosolic calcium (28).

Previous studies have shown that calcium accumulation in other organs such as the heart (8) and skeletal muscle (9) inhibited mitochondrial oxidation and was associated with a lower ATP content of these organs. Recently, Perna et al. (10) reported that a chronic administration of PTH to rats was associated with a rise in resting levels of cytosolic calcium in pancreatic islets and was associated with a decrease in the ATP content of the islets. It is, therefore, plausible to suggest that the rise in cytosolic calcium of the synaptosomes from animals with excess PTH is also responsible, at least in part, for the fall in their ATP content. This in turn affects the function of the calcium-activated ATPase, the Na+/Ca2+ exchange, and the Na+/K+ ATPase, events which would further increase the level of cytosolic calcium. The data of our study showing that prior PTX of CRF rats or the treatment of CRF rats with verapamil corrected the fall in synaptosomal ATP content and the rise in their resting levels of cytosolic calcium are consistent with the postulate regarding the interaction between ATP content, the processes involved in calcium extrusion, and the resting levels of cytosolic calcium.

Our finding of reduced ATP content in brain synaptosomes of rats with CRF are at variance with those reported by others (29,30) in synaptosomes (29) or brain tissue (30) from animals after 45 to 72 h of acute renal failure. The difference between our results and those of others (29,30) is most likely because of a difference in the duration of renal failure. This study showed that the treatment of CRF rats with the calcium channel blocker, verapamil, prevented the increase in the resting levels of cytosolic calcium of brain synaptosomes. This effect could be due to the inhibition of PTH secretion by verapamil, the blocking of PTH-induced calcium entry into synaptosomes by the drug, or both. Several in vivo studies showed either that verapamil does not affect blood levels of PTH (31) or that higher doses of the drug may cause a significant rise in blood PTH levels in normal rats (32,33) or those with moderate renal failure (32). It is reasonable to speculate that the effect of chronic administration of verapamil on the resting levels of cytosolic calcium of synaptosomes of CRF rats was mediated by blocking the calcium channels in the membrane of these structures. Such a proposition would imply that the PTH-induced calcium entry into synaptosomes may directly or indirectly be mediated by the activation of these calcium

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channels that are blocked by verapamil. Although we did not evaluate this issue in the study presented here, Turner and Goldin (34) have demonstrated that rat brain synaptosomes possess active voltage-sensitive calcium channels and that the calcium uptake by these channels is blocked by various calcium channel blockers including verapamil and diltiazem.

It is of interest that verapamil did not affect the resting levels of cytosolic calcium of synaptosomes from normal rats. This finding suggests that the administration of verapamil is effective in situations where the calcium channels are activated and permit greater entry of calcium, such as in the presence of excess PTH. It is also possible that verapamil blocks calcium entry into the synaptosomes of normal rats as well. This reduced calcium movement could be followed by decreased activity of the calcium-activated ATPase because the activity of this enzyme is regulated by calcium concentration. Under such circumstances of reduced entry and decreased extrusion of calcium, the resting levels of cytosolic calcium would not be affected.

We would like to emphasize that the measurements in resting levels of cytosolic calcium and ATP in synaptosomes from animals with excess PTH were measured under certain experimental conditions in vitro and may not reflect in vivo steady-state behavior. However, the studies of synaptosomes from normal animals, those with excess PTH, and those treated with verapamil were conducted under the same in vitro conditions. One, therefore, is justified in suggesting that the differences in cytosolic calcium and ATP between the synaptosomes of these various groups of animals represented the values at the time the synaptosomes were harvested.

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