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ANTINOCICEPTIVE ACTIVITY OF CALCITONIN AND CENTRAL CHOLINERGIC SYSTEM: BEHAVIOURAL AND NEUROCHEMICAL ANALYSES

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Abstract—Behavioural and neurochemical analyses were carried out to investigate the relationship between the antinociceptive activity of porcine calcitonin (pCT) and central cholinergic system in mice and rats. Behavioural studies revealed that the antinociceptive activity of pCT encapsulated in sulphatide-containing liposomes injected intravenously into mice was significantly inhibited by atropine sulphate, but not by atropine methylnitrate, and potentiated by physostigmine, but not by neostigmine. Neurochemical studies using rat brain synaptosomes showed that pCT stimulated synaptosomal sodium-dependent high-affinity choline uptake, which was found to be closely associated with acetylcholine (ACh) synthesis (50–60%). This effect was concentration-dependent. In addition, pCT elicited a biphasic effect on ACh release from synaptosomes with an initial brief period of stimulation and subsequent prolonged inhibition. This stimulation was not affected by atropine sulphate, but markedly reduced by incubation in the presence of diltiazem or in a calcium-free medium, indicating that the modulation of ACh release by the peptide may be mediated by calcium fluxes across the synaptosomal membrane independent of cholinergic receptor activation. However, pCT does not affect the activity of synaptosomal acetylcholinesterase. Therefore, the behavioural study *in vivo* with the neurochemical analysis *in vitro* suggests that the central cholinergic system may be involved in the antinociceptive activity of calcitonin.

Key words: calcitonin; liposomes; antinociception; synaptosomes; choline uptake; acetylcholine release and synthesis

CT†, a polypeptide hormone with 32 amino acid residues arranged in a single chain, is secreted by the C cells in the thyroid gland of mammals or in the ultimobranchial body of non-mammalian vertebrates [1]. In addition to its physiological function involved in calcium homeostasis [1], CT has been shown to exert several central pharmacological activities, notably its analgesic action following its central administration in various species of animals such as rabbit [2], mouse [3, 4], rat [5] and cat [6]. Subarachnoid and epidural administration of CT can induce potent analgesia in patients suffering from chronic, intractable oncological pain [7, 8]. High-density binding sites for CT have been demonstrated in raphe nuclei, periaqueductal grey matter, and spinal neurons, etc. [5, 6, 9], structures involved in the neural pathways related to reception, transmission and modulation of nociceptive information [10]. Our recent study has shown that pCT encapsulated in sulphatide-containing liposomes is able to induce potent central antinociceptive activity upon its systemic administration in mice [11].

Although much work has been done to investigate the central analgesic activity of CT, reports on the mechanism of the CT-induced antinociception are still conflicting. It has been reported that CT antinociception may involve Ca^{2+} fluxes across the neuronal membrane [3, 4], central serotonergic system [12] and catecholaminergic system [13]. Evidence for or against the involvement of endogenous opioids in CT-induced analgesia has also been reported [2, 14–16].

Much evidence shows that the classical neurotransmitter, ACh, plays an important role in central nociceptive regulation [17–20]. It has been observed that the nociceptive stimulation induces an increase in cortical ACh outflow [21]. The central antinociceptive activity produced by some bioactive compounds has been shown to associate with an increase in ACh release in the CNS of animals and humans [19, 20] and the enhancement of ACh turnover rate [18]. Antinociception can be induced by AChE inhibitors [17] or central microinjection of cholinomimetics [22, 23] or by electrical stimulation of cholinergic neurons in specific regions of the brain [24]. This antinociceptive action could be antagonized by AS [17]. Involvement of central cholinergic transmission in the antinociceptive activity of opiate and

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† Abbreviations: CT, calcitonin; pCT, porcine calcitonin; L-pCT, liposome-encapsulated porcine calcitonin; BBB, blood–brain barrier; PC, egg phosphatidylcholine; Chol, cholesterol; BA, buffer A; AS, atropine sulphate; AM, atropine methylnitrate; NS, neostigmine; PS, physostigmine; ACh, acetylcholine; AChE, acetylcholinesterase; Ch, choline; SDHACU, sodium-dependent high-affinity choline uptake; CAT, choline acetyltransferase; VRC, voltage-regulated Ca^{2+} channels.

non-opiate bioactive compounds has been well documented [17, 18, 25]. So far it is not clear whether central cholinergic system is involved in the antinociceptive action of CT.

In view of these considerations, we examined the interrelationship between CT antinociception and central cholinergic system. For this purpose, behavioural studies were performed to examine the influence of peripherally injected cholinergic antagonists and agonists on the antinociceptive activity of pCT encapsulated in sulphatide-containing liposomes administered i.v. in mice. Furthermore, neurochemical analyses were carried out to investigate the modulatory effects of pCT on synaptosomal cholinergic processes in rat brain, including synaptosomal AChE activity, choline uptake, ACh release, and the relationship between synaptosomal choline uptake and ACh synthesis.

MATERIALS AND METHODS

Chemicals

Ficoll was purchased from Pharmacia Fine Chemicals (Sweden). PC, Chol, acetylcholine chloride, acetylthiocholine iodide, physostigmine, neostigmine, BSA, choline chloride, atropine sulphate, atropine methylnitrate, diltiazem, glycylglycine, sodium tetraphenylboron, hemicholinium-3, choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32) and AChE (EC 3.1.1.7) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ATP (sodium salt) was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Butyronitrile was obtained from Fluka (Switzerland). High-purity pCT, as examined by electrophoresis, was kindly provided by Rorer Pharmaceuticals (U.K.). [Methyl-^{3}H]choline chloride (specific activity, 83 Ci/mmol) was from Amersham (U.K.). Universol cocktail was from ICN Biochemicals Inc. (U.S.A.). Sulphatide was prepared as its sodium salt from normal human brain with final purity greater than 98%, as reported elsewhere [11, 26]. All other chemicals used were of analytical grade.

Animals

Both male and female Swiss mice (20–24 g) for behavioural study, and male Wistar rat (200–250 g) for neurochemical study, were obtained from the Laboratory Animals Center of the National University of Singapore. Animals were housed with a constant light–dark cycle at room temperature for at least 3 days prior to the experiment. Food and water were freely available. All animals appeared in good physical condition at the beginning of testing.

Behavioural studies

Preparation of L-pCT. Liposomes were prepared by reverse-phase evaporation method [27], with a composition of PC:Chol:sulphatide at the molar ratio of 7:10:3. These liposomes can transport encapsulated substance that would not otherwise pass through the BBB [11, 28, 29]. Experimental details and liposomal characterization have been reported in our early papers [11, 26]. The final sulphatide-containing liposomes encapsulating pCT (L-pCT) were suspended in buffer A (BA, 5 mM Tris, 20 mM NaCl, pH 7.4) for behavioural test.

Antinociceptive test. Groups (10–14 animals per group) of mice were treated with the following drugs in conjunction with i.v. injection of L-pCT or buffer A prior to the measurement of pain threshold: cholinergic antagonists, AS (1, 6 and 12 mg/kg, i.p., 30 min before i.v. L-pCT) and AM (6 mg/kg, i.p., 30 min before i.v. L-pCT); cholinergic agonists, PS and NS (75 μg/kg, i.p., 1 min before i.v. L-pCT). In each of the above studies, the appropriate controls (buffer A) for each drug treatment were also carried out. Pain threshold was evaluated using the hot-plate test as reported [11]. Briefly, each mouse was placed on a hot plate (50.2°) and the test ended when the animal licked its hind paws. A cut-off time of 40 sec was chosen to prevent tissue damage. Each mouse was tested every 30 min after injection of L-pCT). The degree of antinociception was calculated as a percentage of the maximum possible effect (MPE%) according to the following equation:

$$\text{MPE\%} = (Tt - Tc)/(\text{cut-off time} - Tc) \times 100$$

where Tt is the post-injection (L-pCT) response time and Tc is the pre-injection (L-pCT) control.

Neurochemical studies

Preparation of synaptosomes. Synaptosomes were prepared from whole rat brain using discontinuous Ficoll density gradient [30]. The synaptosomal fraction at the interface between 8% and 12% Ficoll concentrations was harvested for the following studies.

Choline uptake and ACh synthesis by synaptosomes. Choline uptake by synaptosomes was determined by the radioactive method as described by Briggs and Cooper [31]. The experiments were performed in the normal and Na^+-free (Li^+ as substitution) incubation medium, consisting of 3 mM KCl, 1.3 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM glucose, 10 mM Tris, pH 7.4. The final concentration of Na^+ and Li^+ in the uptake medium were, respectively, 140 mM and 0 mM in normal medium and 0 mM and 140 mM in Na^+-free medium. Aliquots (0.1 mL) of synaptosomal suspension (at final concentration of 2.5–3.2 mg protein/mL) in the normal and free-Na^+ medium were mixed with 0.3 ml of incubation medium containing physostigmine (20 μM) in the absence and presence of pCT (0.01–1000 mU/mL, MRC mU/mL), and preincubated for 5 min at 37°. The uptake procedure was started by adding 0.1 mL of choline solution containing [^{3}H]choline (final concentration in the incubation mixture, 5 μCi; free choline, 1 μM) to the sample and continued for 4 min. The reaction was terminated by addition of 1 mL of ice-cold Ca^{2+}-free buffer, and samples were immediately cooled in ice. After centrifugation at 12,500 g for 2 min, the pellet was washed twice with the same buffer and then transferred into a scintillation vial. Universol cocktail (5 mL) was added, and the radioactivity was counted in a Beckman liquid scintillation counter. SDHACU was determined by subtracting the [^{3}H]choline accumulated in synaptosomes incubated and washed in buffer with LiCl instead of NaCl from that in buffer containing NaCl [32].

For determination of ACh synthesis, synaptosomal suspension was incubated with [^{3}H]choline in the normal medium for 8 min. After removing the free [^{3}H]choline by centrifugation, pellets were extracted with 1 M formic acid/acetone (15:85, v/v) [31, 32]. Extracts were dried under nitrogen. The residue was dissolved in normal medium and divided into two equal aliquots for the phosphorylation as described in the determination of [^{3}H]ACh. To one of the aliquotes was added AChE (0.01 U/mL) to hydrolyse the [^{3}H]ACh to [^{3}H]choline. The radioactivity in [^{3}H]ACh was determined by subtracting the dpm value of the AChE-treated aliquot from the corresponding value of the non-treated aliquot [32].

ACh release from synaptosomes. Release of ACh from rat brain synaptosomes was determined according to the method of Briggs and Cooper [31]. For loading synaptosomes with [^{3}H]ACh, synaptosomal suspension containing protein of 9–11 mg/mL was preincubated in normal medium for 5 min and followed by addition of [^{3}H]choline (5 μCi; free choline, 1 μM). After incubation for 30 min, synaptosomes loaded with [^{3}H]ACh were washed three times with Ca^{2+}-free buffer by centrifugation, and the pellet was resuspended in the incubation medium for the release experiment. Neurotransmitter release was assessed by the [^{3}H]ACh discharge from the synaptosomes (final protein concentration of 0.9–1 mg/mL) at 1 min intervals following the addition of pCT. The release medium consisted of the normal medium and physostigmine (20 μM) in the presence of hemicholinium-3 (10 μM) to prevent reuptake of released [^{3}H]choline and further [^{3}H]ACh synthesis. At defined times, the release was stopped by chilling the sample in an ice bath. The supernatant obtained from centrifugation of each sample was kept for [^{3}H]ACh determination.

Determination of [^{3}H]*ACh.* [^{3}H]ACh was separated from [^{3}H]choline by an enzymatic liquid-cation exchange method [31, 32]. Typically, 200 μL sample of supernatant was incubated with 50 mM glycylglycine buffer, pH 8.5, 10 mM ATP, 12.5 mM $MgCl_2$, and 0.01 unit/mL choline kinase in the final volume of 250 μL at 37° for 30 min. The reaction was terminated by chilling the sample in an ice bath. Three hundred microlitres of chilled sodium tetraphenylboron in butyronitrile (10 mg/mL) was added. The tube was thoroughly mixed and centrifuged at a low speed. [^{3}H]ACh was chelated and extracted into the upper organic phase for counting. The [^{3}H]phosphorylcholine remained in the lower aqueous phase and did not contribute to the [^{3}H]-ACh measurement. It has been reported that [^{3}H]-ACh could be quantitatively extracted whereas less than 2% of [^{3}H]choline was extracted following the phosphorylation [31].

Assay of synaptosomal AChE activity. AChE activity was determined by the colorimetric method with acetylthiocholine as substrate [33]. and the chemiluminescence method with the natural substrate, acetylcholine [34], in the absence or presence of pCT at concentrations from 0.01 to 1000 mU/mL.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [35], with BSA as standard.

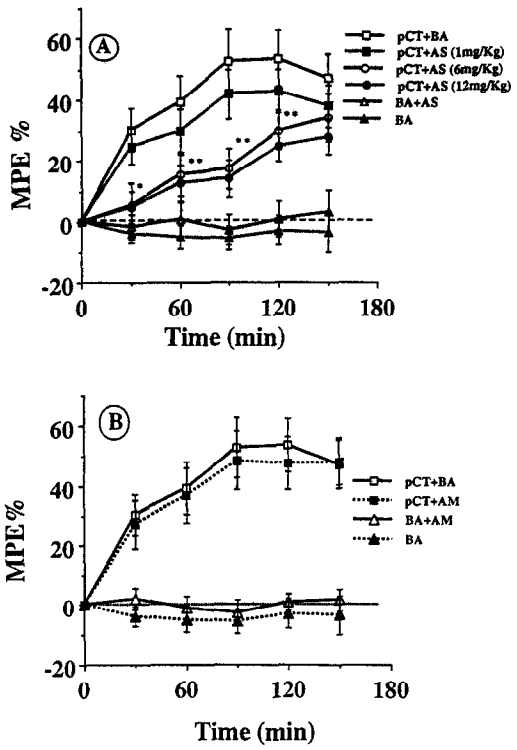

Fig. 1. Effect of cholinergic antagonists on the antinociceptive activity of pCT encapsulated in liposomes (hot-plate test). (A) AS (1, 6, 12 mg/kg); (B) AM (6 mg/kg). Both AS and AM were injected i.p. 30 min before pCT (40 U/kg, i.v.). Data are expressed as MPE%. Each point represents the mean ± SE of 10–14 mice. *$P < 0.05$ versus pCT + BA; **$P < 0.05$ versus BA, BA + AS and pCT + BA.

Data analyses. Data are expressed as means ± SE. Statistical analysis was performed by paired Student's *t*-test. A level of $P < 0.05$ was accepted as statistically significant in all studies.

RESULTS

Behavioural studies

Influence of cholinergic antagonists on the antinociceptive activity of L-pCT. Figure 1 shows the time-course curves of the hot-plate response following the i.v. injection of L-pCT in mice pretreated with AS and AM. It was found that L-pCT (40 U/kg) induced potent antinociceptive activity that maintained at the maximal level for the entire period of observation (2.5 hr), while the control groups injected with BA or free pCT or free liposomes or the mixture of free liposomes and free pCT did not exhibit such an activity [11]. This antinociception induced by L-pCT was partially, but significantly ($P < 0.05$) blocked by atropine sulphate (6 mg/kg) administered i.p. (Fig. 1(A)). There was no significant inhibition at lower dosage of AS (1 mg/kg) and no further enhancement in inhibition upon increase in drug dosage (12 mg/kg). As a comparison, neither BA altered the CT antinociception, nor AS

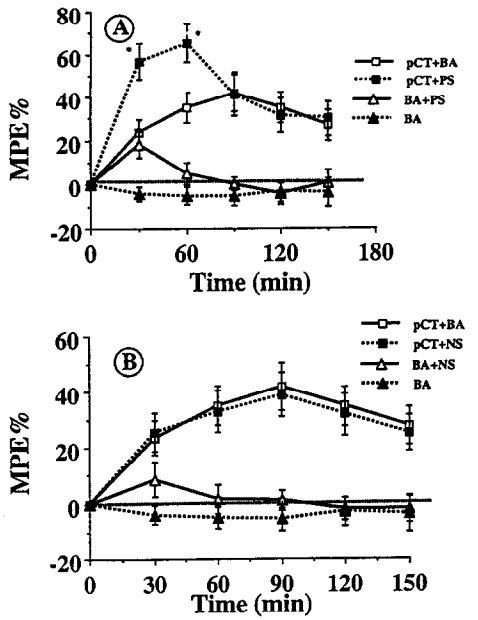

Fig. 2. Influence of cholinergic agonists on the antinociceptive effect of liposome-entrapped pCT (hot-plate test). (A) PS (75 μg/kg); (B) NS (75 μg/kg). Both PS and NS were administered i.p. 1 min before pCT (18 U/kg, i.v.). Data are expressed as MPE%. Each point represents the mean ± SE of 10–14 mice. *$P < 0.05$ versus BA + PS and pCT + BA.

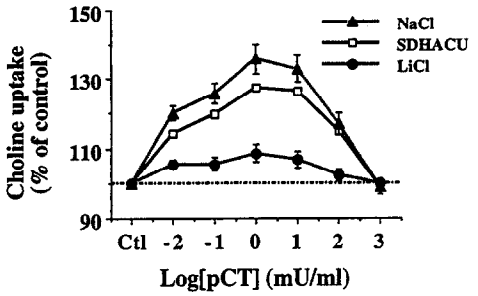

Fig. 3. Effect of pCT on [^{3}H]choline uptake by synaptosomes in the absence (LiCl as substitution) and presence of NaCl upon 4 min incubation. SDHACU was determined by subtracting the [^{3}H]choline accumulated in synaptosomes incubated with LiCl from that in buffer containing NaCl. Values are expressed as percent of control (Ctl), which is defined as 100%. Individual data points represent the means ± SE of four independent experiments.

itself induced any significant change in antinociception. When atropine sulphate and L-pCT were administered simultaneously, the initial onset of inhibition occurred at 60 min. Figure 1(B) shows that the antinociceptive activity of pCT was not affected in animals pretreated with quaternary agent atropine methylnitrate ($P > 0.05$).

Influence of cholinergic agonists on the antinociceptive activity of L-pCT. Further study was carried out to evaluate the influence of cholinergic agonists on the antinociceptive action of pCT (Fig. 2). Physostigmine by itself, at a dose of 75 μg/kg injected i.p., produced a significant antinociceptive effect ($P < 0.05$) with the MPE% of 18% at 30 min. Administered it to animals 1 min before L-pCT, physostigmine enhanced the antinociceptive effect of pCT (18 U/kg) from 23.5% to 56.5% and from 35.2% to 63.5%, respectively (Fig. 2(A)). However, neostigmine did not modify the antinociception of L-pCT (Fig. 2(B)).

Neurochemicalstudies

Effect of pCT on choline-uptake by synaptosomes. Figure 3 shows the dose-response effect of pCT on [^{3}H]choline uptake by synaptosomes in the normal and Na^+-free medium upon incubation for 4 min. The presence of pCT resulted in an increase in the uptake of [^{3}H]choline, which became statistically significant ($P < 0.05$) at pCT concentration of 0.01 mU/mL in the presence of Na^+. The maximum effect was induced by pCT at concentrations of 1 and 10 mU/mL. Then the effect was reduced with increasing concentration of pCT, and almost no increase in [^{3}H]choline uptake was observed at a concentration of 1000 mU/mL of pCT. However, the increase in choline uptake induced by pCT was dependent on the presence of Na^+ and significantly abolished in the Na^+-free medium (Li^+ substitution). The SDHACU was obtained by subtracting the radioactivity accumulated in Na^+-free preparation from the total accumulation in preparation incubated in the normal medium (Fig. 3).

Choline uptake and ACh synthesis. The relationship between synaptosomal [^{3}H]ACh formation and the SDHACU is presented in Table 1. The increase in [^{3}H]choline uptake by synaptosomes induced by pCT was closely associated with enhancement in [^{3}H]ACh formation in the nerve terminals. About 50–60% of the increased [^{3}H]choline uptake through SDHACU induced by pCT at various concentrations (0.01–100 mU/mL) tested was converted to [^{3}H]-ACh with a constant rate under our experimental conditions.

Effect of pCT on [^{3}H]ACh release from synaptosomes. As shown in Fig. 4, pCT induced a biphasic response in ACh release from synaptosomes loaded with [^{3}H]ACh. The relative magnitude of the transmitter release was expressed as percent change from corresponding control. Initial exposure of synaptosomes to pCT resulted in stimulation of ACh release that was of brief duration. Continued incubation with pCT led to the subsequent inhibition of cholinergic neurotransmitter discharge that was substantially greater in duration and magnitude than the initial stimulatory response. For example, [^{3}H]-ACh release was stimulated for about 4 min after initiation of the reaction by pCT (1 mU/mL). In turn, the peptide-mediated inhibition of ACh discharge lasted for greater than 8 min. The peak value for stimulation, occurring at 3 min, and nadir value for inhibition of [^{3}H]ACh release, occurring at 12 min, were 132.3% and 64.4% of control, respectively.

The dose–response effects of pCT on [^{3}H]ACh release from synaptosomes were examined at

Table 1. Relation of [^{3}H]ACh formation to pCT-induced sodium-dependent high-affinity choline (Ch) uptake (SDHACU) by synaptosomes

pCT conc (mU/mL)	Increase in SDHACU (pmol/mg protein)	Increase in ACh formation (pmol/mg protein)	Ratio of ACh/Ch
0.01	2.55 ± 0.30	1.40 ± 0.12	0.55
0.1	3.60 ± 0.25	1.81 ± 0.20	0.50
1	4.80 ± 0.51	2.69 ± 0.21	0.56
10	4.65 ± 0.41	2.71 ± 0.30	0.58
100	2.70 ± 0.29	1.46 ± 0.16	0.54

Data are obtained by substracting the control value from that in the presence of pCT and expressed as means ± SE of four determinations upon 8 min incubation.

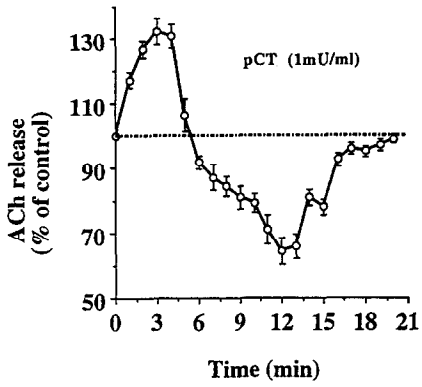

Fig. 4. Time course of [^{3}H]ACh release from synaptosomes induced by pCT (1 mU/mL). Results for [^{3}H]ACh release are expressed as percent of corresponding control (100%). Each point represents the mean ± SE of four separate experiments.

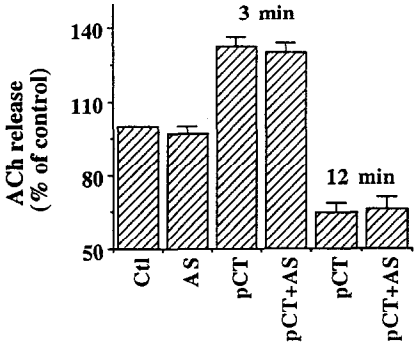

Fig. 6. Effect of AS (10^{-6} M) on synaptosomal [^{3}H]ACh release induced by pCT (1 mU/mL) upon 3 and 12 min incubation. Values are expressed as percent of corresponding control (Ctl, 100%). Data depicted by bar and vertical line represents mean ± SE of three to four different experiments. AS did not significantly influence the [^{3}H]ACh release from synaptosomes induced by pCT.

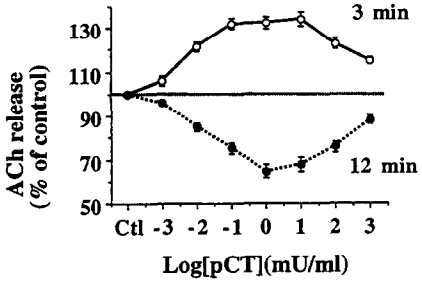

Fig. 5. Concentration-dependent biphasic effects of pCT on synaptosomal [^{3}H]ACh release. The experiments were performed upon 3 (peak) and 12 min (nadir) incubation. Values are expressed as percent of corresponding control (100%). Each point represents the mean ± SE of four different determinations.

concentrations ranging from 0.001 to 1000 mU/mL (Fig. 5). Peak and nadir values were normalized to the percent of corresponding control. The biphasic response of stimulation followed by inhibition of [^{3}H]ACh release was observed throughout the range of pCT concentrations examined. The maximal effects for both stimulation and inhibition of [^{3}H]-ACh release were induced by pCT at concentrations of 1 and 10 mU/mL.

Influence of atropine sulphate and diltiazem on pCT-induced [^{3}H]*ACh release from synaptosomes.* To assess the possible mechanism by which pCT may exert its action on cholinergic neurotransmission, experiments were carried out with cholinergic antagonist, AS and calcium channel blocker, diltiazem. Additional experiments were performed by using a release medium containing no added calcium. Atropine sulphate, when added to the incubation medium at a concentration of 1 μM, did not influence either basal or pCT stimulated [^{3}H]-ACh release (Fig. 6). On the other hand, diltiazem (10^{-4} M) significantly reduced the [^{3}H]ACh release stimulated by pCT (1 mU/mL) (Fig. 7). The similar response to pCT was also observed by removal of calcium from the release medium (Fig. 7).

The effect of pCT on the activity of synaptosomal AChE. This was estimated at different pCT concentrations. The results obtained by Ellman's

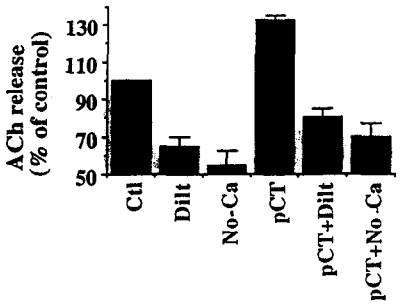

Fig. 7. Influence of Ca^{2+} and diltiazem (Dilt, 10^{-4} M) on synaptosomal [^{3}H]ACh discharge induced by pCT (1 mU/mL) upon 3 min incubation. Results are expressed as percent of control (Ctl, 100%). Each value illustrated by bar and vertical line represents the mean ± SE of three to four separate determinations.

Table 2. Effect of pCT on the activity of synaptosomal AChE (mean ± SE, N = 4)

CT conc (mU/mL)	AChE activity (μmol/min per g of protein)	P value
0 (Control)	40.2 ± 1.4	
0.01	40.4 ± 1.5	> 0.05
0.1	39.4 ± 1.0	> 0.05
1	40.5 ± 1.3	> 0.05
10	37.5 ± 1.1	> 0.05
100	40.1 ± 1.5	> 0.05
1000	39.7 ± 1.2	> 0.05

method indicated that pCT at concentrations between 0.01 and 1000 mU/mL did not influence the AChE activity (Table 2), even when pCT was preincubated with synaptosomes for as long as 15 min, or with the addition of 0.1 mM Ca^{2+} to the reaction mixture ($P > 0.05$). The same result was obtained with ACh as substrate assayed by chemiluminescence method.

DISCUSSION

Our early investigation has shown that systemic administration of pCT encapsulated in sulphatide-containing liposomes is able to induce potent central antinociceptive activity in mice [11]. This observation could be attributed to the ability of these vesicles to pass through the BBB [29]. The present behavioural study reveals that the antinociceptive action of such CT is influenced by cholinergic antagonist and agonist. The antinociceptive effect of pCT encapsulated in sulphatide-containing liposomes was potentiated by i.p. injection of physostigmine, but not by neostigmine, because the latter is not able to pass through the BBB [17]. This antinociception was significantly blocked by i.p. administration of AS. However, atropine methylnitrate, which does not pass through the BBB [22], did not affect the antinociceptive activity of pCT. These biological studies indicate that the central but not peripheral cholinergic system is associated with the CT antinociception. This hypothesis has also been recently suggested by Braga *et al.* [36] and is supported by the present neurochemical studies *in vitro*, which show that pCT stimulates synaptosomal choline uptake and ACh synthesis, and induces ACh release from nerve endings.

Possibly, antinociceptive action of CT would also result from an inhibition of AChE activity, thereby increasing synaptic content of ACh. Present study shows that pCT, at doses ranging from 0.01 to 1000 mU/mL, even using natural substrate, has no effect on the activity of synaptosomal AChE *in vitro*. This is in agreement with the observations that the AChE activity of rat brain was not influenced by pCT *in vivo* [37]. Therefore, it is unlikely that the antinociceptive activity of CT involving cholinergic neurotransmission could be due to its inhibition of the AChE.

With respect to the relationship between the central cholinergic system and CT antinociception, studies in rodents and other mammals have shown that the regional distribution of cholinergic neurons [17, 38, 39] largely parallels that of CT binding sites [5, 9, 29] in the CNS, such as brainstem and dorsal horn of the spinal cord. Interestingly, the antinociceptive activity could be induced in animals upon central microinjection of either cholinomimetics or CT into pontine region and periaqueductal grey matter [5, 6, 17, 23]. This indicates that there may be some relationships, such as the common pathways involved, in antinociception induced by cholinergic drugs and CT. Although Sherriff *et al.* failed to show a continuous cholinergic projection from the brainstem to the spinal cord in rat [40], several descending cholinergic pathways from the raphe nuclei to spinal cord have been demonstrated [41] and cell bodies staining for CAT have been detected in the spinal dorsal horn [20]. It is possible that CT may interact with its receptors located in the specific sites in brainstem or spinal cord [5, 6] to activate the descending cholinergic pathways, leading to the inhibition of the nociceptive transmission. It appears there are some neurochemical changes in the central cholinergic system, such as release of ACh as found in this study, which might mediate the antinociceptive action of CT as measured in the hot-plate test. This suggests that at least some CT activity might be dependent upon its action on cholinergic neurons. On the other hand, because of some differences in regional distributions of cholinergic projections and CT receptors in the CNS [5, 9, 17, 39], it seems that activation of cholinergic neurons might only partially account for antinociceptive action of CT. Some other possibilities are that CT may stimulate non-cholinergic descending inhibitory systems through opiate [15, 16] and other neurotransmitter mechanisms such as serotonin [12] and catecholamines [13], as suggested. In addition, central nicotinic cholinergic receptors may play a role in CT analgesia since nicotinic receptors are present in the CNS and notably found on certain polysynaptic sensory afferent pathways [20]. These may explain why relatively high doses of AS can only partially inhibit

the antinociceptive activity of pCT encapsulated in liposomes as observed in this investigation.

The association between SDHACU and ACh synthesis has been extensively studied [32, 42, 43]. It has been postulated that SDHACU is related to rate-limiting reaction for ACh synthesis in cholinergic nerve endings, and ACh formation can be regulated by factors influencing SDHACU [42, 43]. In our study, it was found that pCT enhanced the Na^+-dependent choline uptake by synaptosomes. Relatively constant amounts (50–60%) of increased choline accumulation stimulated by pCT was converted to ACh. Because pCT initially stimulated ACh release, the actual conversion should be higher than that measured. Thus, our study is consistent with the investigation by others [42] that the percent of choline taken up by the high affinity system which is then converted to ACh is quite constant (60–70%) in synaptosomes under a variety of experimental conditions. However, some investigators reported that all of the choline entering the nerve endings via SDHACU is rapidly converted into ACh because of the presence of large excess of CAT relative to the substrate [32]. In addition, it has been suggested that SDHACU and ACh formation are coupled processes in the cholinergic neurons. An increase in the rate of one function drives a higher rate in the other [32, 42]. ACh release appears to be the major prerequisite for acceleration of SDHACU and ACh formation [32, 42]. Present study shows that pCT stimulates the ACh release, leading to the reduction of cytoplasmic ACh, and then choline acetylation would be stimulated because studies *in vitro* and *in vivo* show that stimulation of ACh release results in an increase in ACh formation [42, 43]. The formation of ACh could cause a lower level of cytoplasmic choline which in turn would stimulate the choline uptake. It is conceivable that the increase in SDHACU induced by pCT might be due to its direct activation of SDHACU, or stimulated by the ACh release, leading to the enhancement in the supply of choline for acetylation. Therefore, the present investigation supports the hypothesis that choline uptake is closely associated with the synthesis of ACh, especially when the neurotransmitter release is increased [32, 42, 43].

The mechanism involved in the stimulatory effect of CT on ACh release from the nerve endings is not clear. It appears not due to its direct action on the cholinergic receptors because AS, the cholinergic receptor blocker, does not affect the ACh release induced by the peptide as found in the present investigation. However, ACh release induced by pCT seems to be mediated by Ca^{2+} fluxes across the synaptic membrane. Studies show that CT is able to enhance Ca^{2+}-dependent potentials in parasympathetic neurons [44], stimulate Ca^{2+} influx [45], and induce an increase in synaptosomal free intracellular Ca^{2+} level [4]. Such CT-induced increase in free Ca^{2+} concentration has been shown to associate with the influx of calcium via voltage-regulated calcium channels [4]. Since the calcium entry has been reported to relate to the altered release of the neurotransmitters from nerve endings [46], it is conceivable that change in free synaptosomal Ca^{2+} concentration induced by pCT would be

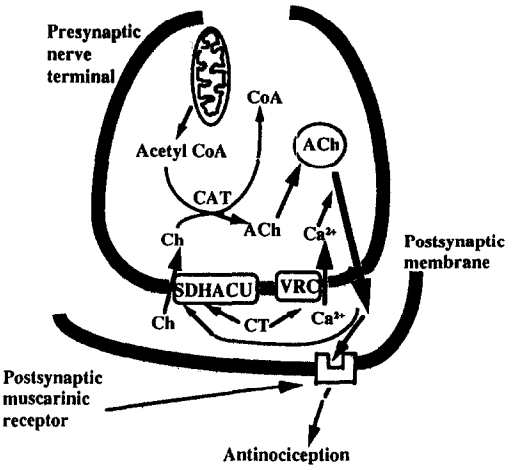

Fig. 8. A model of postulated mechanisms on the modulation of cholinergic activities at nerve terminals induced by pCT. Porcine CT stimulates SDHACU, resulting in an increase in ACh synthesis catalysed by choline acetyltransferase (CAT), and induces Ca^{2+} influx by voltage-regulated Ca^{2+} channels (VRC), evoking the release of ACh which, in turn, stimulates the SDHACU. The released ACh acts on the postsynaptic cholinergic receptors, inducing antinociception.

expected to result in the release of ACh. This hypothesis is also supported by the results as found in this study that pCT-stimulated ACh release was inhibited by incubation in the presence of voltage-regulated Ca^{2+} channel blocker (diltiazem) or in the Ca^{2+}-free medium. It is interesting to note the inverted U-shaped effect–concentration curves obtained with pCT for choline uptake (Fig. 3), stimulation and inhibition of ACh release (Fig. 5) or ACh synthesis (Table 1). Decrease in these biological effects caused by pCT at higher concentrations may be due to the down-regulation of CT receptors in the synaptic membrane [47]. They may be relevant to the therapeutical use of CT.

With respect to the significance of the biphasic action of pCT on ACh release, stimulation of ACh release from synaptosomes may be related to the CT analgesia. The discrepancy between a short period (5 min) of ACh release from nerve endings *in vitro* (Fig. 4) and a long period of analgesic effect (more than 3 hr) *in vivo* (Figs. 1 and 2) also suggests the partial involvement of cholinergic system in CT antinociception as discussed earlier. The prolonged inhibition of ACh release from nerve terminals may be associated with other biological activities of CT such as its inhibition of gastric acid secretion and gastric motility [1, 48]. These effects have been shown to relate to a decrease in the cholinergic vagal efferent nerve activity induced by CT [48]. The biphasic phenomenon was also observed in synaptosomal calcium fluxes produced by CT and CGRP as found by us (unpublished data) and others [45], and in cholinergic neuronal ACh discharge caused by calcitonin gene-related peptide [49].

In summary, the present behavioural studies show

a reduction by the cholinergic antagonist and potentiation by the cholinergic agonist in the antinociceptive effect of pCT encapsulated in liposomes These biological responses *in vivo* are consistent with the neurochemical analyses *in vitro* that pCT stimulates synaptosomal choline uptake and ACh synthesis, and induces the ACh release from nerve terminals. A model of postulated mechanisms on the modulation of cholinergic processes induced by pCT at nerve endings is shown in Fig. 8. The results obtained support the hypothesis of the possible involvement of central cholinergic system in the antinociceptive action of CT. Finally, it is necessary to point out that the hot plate test for the antinociception has one drawback: it records a complex motor reaction to the nociceptive stimulus, despite its wide use in pain studies. ACh is an ubiquitous transmitter in the CNS and has multiple functions. The relationship between the CT-induced ACh release and motor system and influence of motor effect on the analgesic response remain unclear. Further studies are required to clarify the precise relationship between the modulation of neurotransmitters in the CNS and the antinociceptive activity of calcitonin *in vivo*.

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