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Salmon Calcitonin-Induced Modulation of Free Intracellular Calcium

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WELCH, S. P. AND K. G. OLSON. *Salmon calcitonin-induced modulation of free intracellular calcium.* PHARMACOL BIOCHEM BEHAV **39**(3) 641–648, 1991.—Salmon calcitonin (sCT), a hormone shown to modulate calcium in the periphery modulated free, intracellular calcium, ($[Ca^{++}]i$), in mouse brain synaptosomes as measured by changes in fura-2-mediated fluorescence. A 5-min incubation of synaptosomes with sCT produced an increase in the basal levels of $[Ca^{++}]i$ and an increase in KCl-stimulated levels of $[Ca^{++}]i$. A 5-min pretreatment of mice with intraventricularly administered sCT antagonized morphine-induced antinociception in the tail-flick test, and facilitated naloxone antagonism of morphine. Conversely, pretreatment of synaptosomes for 1 h with salmon CT produced a decrease in depolarization-stimulated levels of $[Ca^{++}]i$. The sCT-induced decrease in the stimulated rise in $[Ca^{++}]i$ at 1 h correlated temporally to sCT-induced antinociception in vivo. The effects of sCT in the electrically stimulated guinea pig ileum bioassay appeared to correlate to sCT effects in vivo. The data indicate that calcitonin may function as a neuromodulator via modulation of Ca^{++} within the central nervous system.

Salmon calcitonin Intracellular calcium Synaptosomes Opiate antinociception

CALCITONIN (CT), a hormone secreted by the thyroid, is involved in the maintenance of Ca^{++} homeostasis in the periphery (43). CT causes a decrease in serum Ca^{++} levels and sequesters Ca^{++} in bone. Studies done with a variety of peripherally derived cells in culture clearly indicate that CT facilitates $^{45}Ca^{++}$ uptake (3, 28, 44). CT also increases mitochondrial calcium uptake, an effect which may account in part for calcium sequestration by CT in bone (10). The role of CT in the central nervous system (CNS) is less well established. Autoradiography has shown that CT binding sites occur within the CNS, especially in areas that modulate pain and its transmission (14, 22, 27, 32, 46). Intraventricularly (ICV)-administered sCT interacts with a wide array of neurotransmitter and hormonal systems, thus producing a variety of effects such as anorexia, decreased water intake (20, 51, 59), decreased locomotor activity (2,57), inhibition of gastric acid secretion (42) and antinociception, which has both nonopiate-like (49) and opiate-like characteristics (62). CT has been shown to increase the release of beta-endorphin in humans (19,36). In addition, leu enkephalin appears to regulate the secretion of CT (23). These data indicate that CT/opioid interactions occur. In addition, CT has been shown to attenuate the withdrawal signs following chronic opiate administration (7). Calcitonin has been postulated to exert its varied effects in the CNS via neuronal modulation of calcium fluxes (14, 37, 42, 63). CT has recently been shown to block thyrotropin-releasing-hormone-induced increases in intracellular calcium in pituitary cells subsequent to a decrease in prolactin secretion (13).

The role of Ca^{++} in opiate antinociception has been shown by many investigators. Morphine-induced antinociception can be blocked by ICV $CaCl_2$, as well as the ionophore A23187, and can be potentiated by Ca^{++} chelators (5, 26, 31, 60). Morphine has also been shown to decrease $^{45}Ca^{++}$ uptake to synaptosomes (13, 25, 35). Previous investigations in our laboratory have shown that sCT pretreatment of mice for 2 h enhances the antinociceptive effects of morphine (62). We hypothesized that sCT might modulate opiate antinociceptive effects by modulation of intracellular calcium. Therefore, the purpose of this work was to determine whether sCT-induced alterations of $[Ca^{++}]i$ in synaptosomes could be correlated to the intrinsic antinociceptive effects of sCT in vivo previously demonstrated in our laboratory (62), and to sCT-induced modulation of opiate antinociception. We also investigated the effects of sCT alone, and in combination with morphine, in the electrically stimulated guinea pig ileum (GPI) bioassay in order to determine whether the modulatory effects of sCT in vivo also occurred in this isolated organ preparation.

METHOD

Synaptosomes were prepared from mouse brain using subcellular fractionation techniques described by McGovern et al. (40). Male Swiss Webster mice were decapitated, and the whole brain, minus cerebellum, was homogenized in 0.32 M sucrose in Kreb's buffer (pH 7.4–7.5) on ice (1 gram of tissue/20 ml sucrose). The Kreb's buffer was composed of (mM): NaCl 120;

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KCl 5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 1; glucose 10, $NaHCO_3$ 20, and was brought to pH 7.5 with 1 N HCl. The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was removed and centrifuged at 18,000 × g for 20 min. The pellet was resuspended in 16% Ficoll (16% w/v Sigma Type 400 dialyzed Ficoll) which was prepared in 0.32 M sucrose. A 7.5% Ficoll suspension was layered by hand over the 16% Ficoll/pellet suspension to form a discontinuous gradient. The discontinuous gradient thus formed was centrifuged for 1 h at 40,000 × g using a Sorvall RC2-B refrigerated ultracentrifuge. The synaptosomes which banded at the 16%/7.5% Ficoll interface were removed by aspiration and washed with a 1:1 sucrose (0.32 M)/Kreb's buffer solution. The synaptosomes were centrifuged for 5 min at 18,000 × g and washed again in 1:2 sucrose/Kreb's buffer solution and again centrifuged for 5 min at 18,000 × g.

The resultant pellet was resuspended in 23 ml of buffer (well oxygenated), divided into 2-ml aliquots, and loaded with 3 μM fura-2/AM (Molecular Probes, Inc.) which was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the tube was 1%. Fura-2/AM hydrolysis appears to be greatly enhanced when small aliquots of the synaptosomes are used for fura loading. The aliquots were then incubated for 60 min at 37°C in a shaking water bath and were vortexed following 30 min incubation. At the end of the incubation, the synaptosomes were centrifuged for 5 min at 18,000 × g and washed twice in Kreb's buffer solution and again centrifuged for 5 min at 18,000 × g. The synaptosomes (pellet) were resuspended in oxygenated Kreb's buffer for the measurement of intracellular Ca^{++}. The protein concentration was 0.8–1 mg/ml as determined by the Lowry method (39). The Ficoll and buffers were prepared fresh prior to each assay and kept on ice. Synaptosomes were kept on ice until they were used.

The SPEX Model CM-3/System (SPEX Industries, Inc., Edison, NJ) was used to measure the free calcium within the synaptosomes. The fluorescent dye fura-2/AM (as the acetoxymethyl ester) does not bind calcium and is excited by light with a wavelength of 380 nm. The fura-2 ester is hydrolyzed within the cell or synaptosome, and the free fura-2 then binds Ca^{++}. Bound fura-2 is excited by light at 340 nm. The ratio of excitation intensities of fura-2 at both intensities (340/380) was used to calculate $[Ca^{++}]i$ at 505 nm emission. The fura-2 signal in the presence of a 100-fold excess of EGTA (prepared in 3 M Tris, pH 8.3) was determined to give a background value of relative fluorescence (or F_{min}). The maximal fluorescence (F_{max}) was determined when fura-2 was maximally bound to Ca^{++} following lysis of the synaptosomes with 0.1% Triton X-100 and addition of a 2-mM concentration of Ca^{++}.

The F_{max} and F_{min} were determined for each synaptosomal preparation according to the method of Komulainen and Bondy (34). In addition, a separate synaptosomal preparation was incubated with 1% DMSO instead of fura-2/AM. These synaptosomes which were not fura-2 loaded were used for the quantification of autofluorescence. The autofluorescence of all drugs was also examined in these unloaded synaptosomes. Correction of the signals at 340 and 380 nm for autofluorescence preceded determination of the 340/380 ratio. In addition, each synaptosomal preparation was evaluated for dye leakage by the addition of $MnCl_2$ (20 μM) following a 1-min, 5-min, or 60-min incubation of the synaptosomes with fura-2/AM. The leakage was calculated, and the calcium concentrations were corrected accordingly. From the corrected 340/380 ratio, the free calcium concentration was determined using the method of Grynkiewicz et al. (24) using 224 nM as the K_D for fura-2 and the following equation where F = the fluorescence of the sample, and Sf2 and Sb2 denote the fluorescence at 380 nm in calcium-free and saturating calcium conditions, respectively:

$$[Ca^{++}]_i = K_D \times \frac{(F-F_{min})}{(F_{max}-F)} \times \frac{Sf2}{Sb2}$$

Each experiment was run in duplicate and repeated using 3 to 6 separate sets of synaptosomes. The average calcium concentrations and percent of change in calcium concentrations were determined along with the standard error of the mean (S.E.M.). The mean for each drug treatment was compared to that of the control using Student's *t*-test.

sCT (Bachem), morphine sulfate (Malinckrodt, Inc.), and naloxone hydrochloride were prepared in distilled water. Nifedipine (Sigma Chemicals) was prepared in DMSO vehicle. All drugs and vehicles were added to 2 ml of the synaptosomal preparation in 20-μl aliquots. Appropriate vehicle controls were also evaluated. The synaptosomes were depolarized by the addition of a 50-mM KCl/Kreb's buffer. A continuous, real-time graph of the changes in fluorescences was obtained prior to and following all drug additions. The synaptosomes were equilibrated for 5 min with stirring at 37°C prior to the addition of any drug or vehicle. The synaptosomes that were incubated for 1 hour in well-oxygenated buffer had somewhat higher resting basal calcium concentrations. Stirring was only performed for 5 min prior to the measurement of basal calcium following the 1-h incubation. The average leak at 1 min was 22 nmol calcium. At 5 min, this leak was 35 nmol calcium, but increased to 85 nmol calcium following a 60-min incubation. When corrected for leakage, the basal calcium levels at 1 hour were still significantly higher than following a short incubation. sCT-induced antinociceptive effects are maximal in vivo at 2 h after intraventricular injection. However, sCT-induced antinociception has been demonstrated in our laboratory at 1 h after ICV administration of the drug [Welch et al. (62)]. The 2-hour incubation of the synaptosomes in vitro yielded a preparation with greater than 100 nmol calcium leakage and a lack of response to KCl stimulation. For these reasons, this time point was not used in subsequent studies.

A time-course study was performed to determine the time of the peak effect of morphine on KCl-stimulated $[Ca^{++}]i$. The results of this study (not shown) indicated that morphine produced the greatest attenuation of KCl-stimulated $[Ca^{++}]i$ following a 2-min incubation with the synaptosomes at 37°C. This pretreatment time was used in all studies with morphine.

Electrically Stimulated Guinea Pig Ileum Bioassay

The protocol was similar to that of Paton (48) as modified by Harris et al. (29). Male albino guinea pigs (Dominion Laboratories, Dublin, VA) weighing 200–400 g were sacrificed via decapitation, and 2-cm strips of ileum were removed and placed in an oxygenated Kreb's buffer, pH 7.5. The content of the Kreb's buffer was as follows: (mM) NaCl 137; KCl 2.7; $MgSO_4$ 1; NaH_2PO_4 0.36; $NaHCO_3$ 11.9; $CaCl_2$ 1.35; dextrose 11.1. Segments of ileum were connected via surgical suture to a force transducer such that tension on each segment was 1 g. The strips of ileum were constantly oxygenated and bathed with 37°C Kreb's buffer in a 10-ml glass bath. A Grass polygraph was used to record the contractile responses of the ileum in response to a discontinuous pulse current of 30 V every 10 s. The tissue was allowed to equilibrate for 1 h prior to the addition of drugs. Each experiment consisted of three separate strips of ileum. One strip received sCT only; one strip received morphine only; and one strip received sCT at 5 min, 1 h or 2 h prior to morphine. The dose-response curve of morphine was generated in the strip receiving morphine only and also in the strip incubated with sCT prior to morphine. Three or more concentrations of morphine were used to generate a morphine dose-response curve. Each

TABLE 1

THE EFFECTS OF sCT ON BASAL AND KCl-STIMULATED INCREASES IN FREE INTRACELLULAR CALCIUM IN SYNAPTOSOMES

Treatment	Basal $[Ca^{++}]i$	KCl-Stimulated $[Ca^{++}]i$	% Change From Basal
Vehicle 60 min inc. N=6	371(58)*	458(71)	25(5)
sCT 10^{-6} M 60 min inc N=6	320(71)	333(55)	9(6)*

Synaptosomes were prepared as previously described using fura-2/AM. After a 5-min equilibration period at 37 °C, various drug or vehicle was incubated with the synaptosomes for the length of time indicated in the table. The changes in basal $[Ca^{++}]i$ were determined. The synaptosomes were depolarized with 50 mM KCl, and the percent change in intracellular calcium (from the basal level) was determined. The average calcium concentration, standard error (shown in the parentheses), and the statistical significance were determined as described in the Method section. In each treatment group the "N" indicates the number of separate preparations of synaptosomes that were tested.

*$p<0.05$ from the appropriate vehicle control.

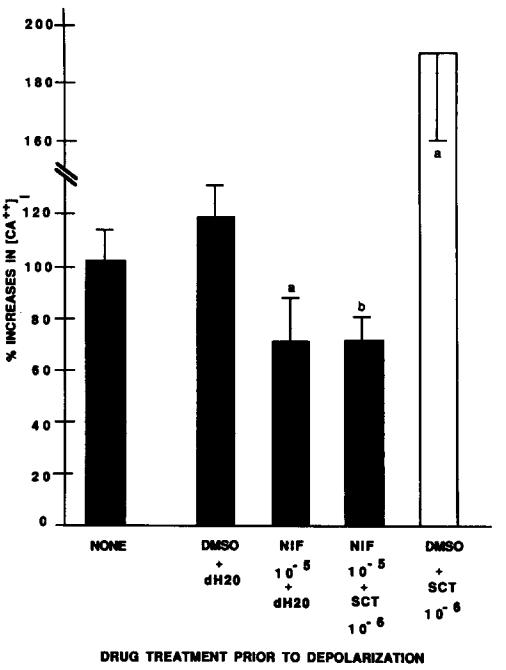

FIG. 1. The effects of sCT and nifedipine, alone and in combination, on KCl-stimulated levels of free intracellular calcium in synaptosomes. Synaptosomes were prepared as previously described using fura-2/AM. After a 5-min equilibration period at 37°C, sCT (1.0 μM) or its vehicle (distilled water) was incubated with the synaptosomes for 5 min alone or in combination with DMSO or nifedipine. The changes in KCl-stimulated $[Ca^{++}]i$ were determined. The average percent change, standard error, and the statistical significance were determined as described in the Method section. In each treatment group, 4–17 individual samples were evaluated. These samples were generated from at least 4 separate preparations of synaptosomes (n=4). (a) $p<0.05$ from the appropriate vehicle control. (b) $p<0.05$ from appropriate vehicle control and from sCT alone.

experiment was repeated at least three times. Naloxone (1 μM) was added to all three strips following the determination of maximal sCT- and morphine-induced inhibition of the contraction of the ileum to test for reversibility of the inhibition. Strips of ileum were used only once. The average percent inhibition, along with the standard error of the mean (S.E.M.), was determined for each treatment group. The mean for each treatment was compared to control using Student's *t*-test. The concentrations of sCT and naloxone were chosen in approximately the same range used for the calcium experiments.

Tail-Flick Test

Drugs were injected free-hand into the lateral ventricles of the brains of male Swiss Webster mice (Dominion Laboratories, Dublin, VA) weighing 20–30 g under light ether anesthesia following a transverse incision of the scalp using the method of Pedigo et al. (50). Injection volumes were 5 μl per mouse of either vehicle (distilled water) or sCT prepared in distilled water. Animals receiving vehicle (ICV) or sCT (ICV) were then injected subcutaneously (SC) either 5 min or 2 h later with morphine sulfate in a volume of 0.1 cc/10 g body weight. The time points chosen (5 min and 2 h) for pretreatment with sCT or vehicle are the time points of maximal sCT-induced blockade (5 min) or facilitation (2 h) of the morphine response. Salmon-CT-induced antinociceptive effects, although observed at 1 h, are maximal at 2 h after ICV administration (61). Twenty minutes after morphine was injected SC, the mice were tested in the tail-flick test. Some animals were injected concurrently with sCT (0.0016 μg, ICV) and naloxone (0.01 mg/kg, SC) 5 min prior to morphine (SC) to test for sCT-induced facilitation of the naloxone antagonism of morphine. The animals were tested for antinociception 20 min following morphine administration using the tail-flick test. The tail-flick procedure was that of D'Amour and Smith (9) as modified by Dewey et al. (11). Control reaction times of 2–4 s and a cut-off time of 10 s were employed. Antinociception was quantified as the % MPE (% maximum possible effect) as developed by Harris and Pierson (30). The following formula was used to calculate % MPE:

$$\%\text{MPE} = 100 \times \frac{\text{test} - \text{control}}{\text{cut-off time} - \text{control}}$$

The % MPE was calculated for each mouse using 6–12 mice per dose. Using the % MPE for each mouse, the mean effect and S.E.M. were calculated for each dose. The means were compared using Student's *t*-test. Dose-response curves consisting of at least three doses were generated for morphine in the presence of vehicle or sCT pretreatment. ED_{50} values and 95% confidence limits (CLs) were determined for morphine using the method of Litchfield and Wilcoxon (38).

RESULTS

Effects of sCT on $[Ca^{++}]i$

Basal $[Ca^{++}]i$ in untreated synaptosomes was 285 ± 43 nmol. The increase in $[Ca^{++}]i$ induced by 50 mM KCl (depolarization) (Fig. 1) was 105%. This increase was significantly reduced to a 71% rise by the addition of nifedipine (10 μM) prepared in DMSO vehicle, but not DMSO vehicle plus distilled water vehicle in combination (118% rise). The addition of salmon CT significantly enhanced depolarization-induced rises in intracellular

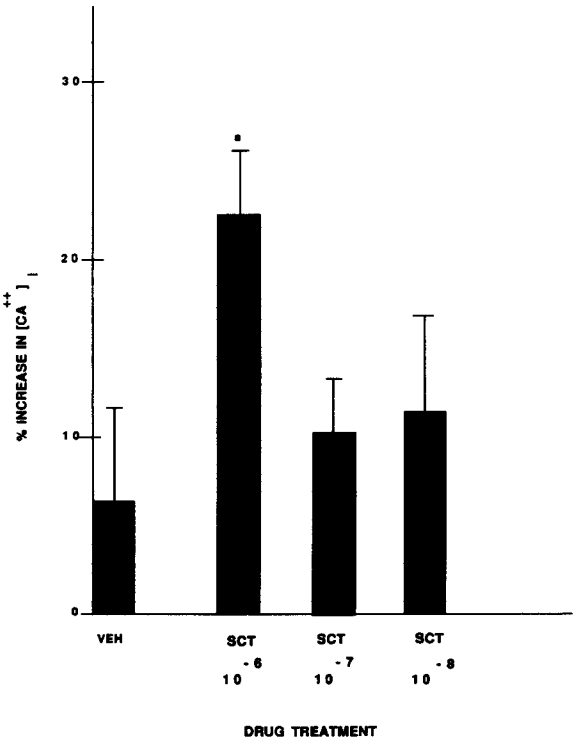

FIG. 2. Effects of sCT on basal free intracellular calcium in synaptosomes. Synaptosomes were prepared as previously described using fura-2/AM. After a 5-min equilibration period at 37°C, sCT or vehicle was incubated with the synaptosomes for 5 min. The changes in basal $[Ca^{++}]i$ were determined. The average percent increase in calcium concentration, standard error, and the statistical significance were determined as described in the Method section. In each treatment group, at least 7 individual samples were evaluated. These samples were generated from at least 4 separate preparations of synaptosomes (n=4). (a) $p<0.05$ from the appropriate vehicle control (5-min incubation).

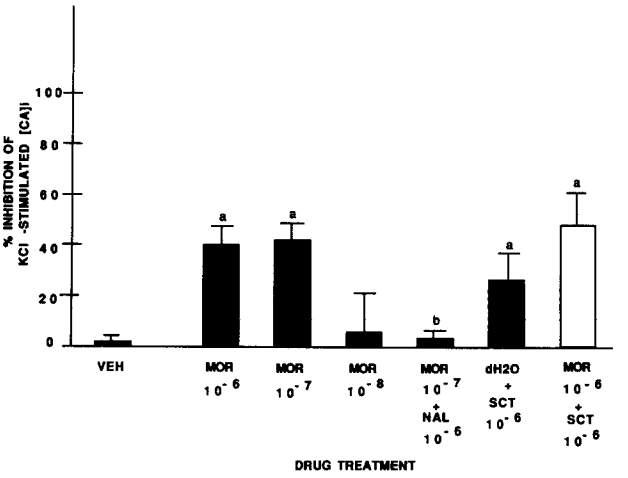

FIG. 3. Effects of morphine, alone and in combination with naloxone and sCT, on depolarization-induced increases in free intracellular calcium in synaptosomes. Synaptosomes were prepared as previously described using fura-2/AM. After a 60-min incubation period at 37°C with sCT or vehicle, naloxone and morphine were added to the preparation at 2 min prior to depolarization. The changes in basal $[Ca^{++}]i$ were determined. The synaptosomes were depolarized with 50 mM KCl, and the percent increase in intracellular calcium (due to the addition of the KCl) was determined. The average percent KCl-induced increase in calcium concentration in the presence of the vehicle only was determined. The percent inhibition of that increase by sCT, morphine, naloxone plus morphine, or sCT plus morphine was determined. Standard errors and the statistical significance were determined as described in the Method section. In each treatment group, at least 7 individual samples were evaluated. These samples were generated from at least 4 separate preparations of synaptosomes. (a) $p<0.05$ from the appropriate vehicle control. (b) $p<0.05$ from morphine (0.1 μM).

calcium (raising calcium levels to 184% of baseline), an effect attenuated by pretreatment with nifedipine. The enhanced calcium entry in the presence of sCT is most likely due to the entry of calcium through voltage-regulated calcium channels. However, these data do not rule out the possibility that the nifedipine produces an effect which cannot be overcome by the addition of the sCT.

Salmon calcitonin (1 μM) produced a significant increase in basal $[Ca^{++}]i$ (Fig. 2). This increase was not observed immediately upon addition of the sCT to the synaptosomes, but was due to a gradual increase in $[Ca^{++}]i$ over the 5-min time course, which was not due to enhanced dye leakage. No greater increases in $[Ca^{++}]i$ were observed after the 5-min incubation. The increase in basal $[Ca^{++}]i$ did not occur in the absence of extracellular calcium, and was thus presumed to occur via calcium influx. Lower concentrations of sCT did not significantly alter basal $[Ca^{++}]i$, possibly due to the use of the whole brain for the preparation of the synaptosomes, which would preclude the measure of changes in basal $[Ca^{++}]i$ in discrete brain regions.

Incubation of the synaptosomes for 1 h with distilled water vehicle produced a rise in the basal intracellular levels of calcium to 371±58 nmol (Table 1) from the 285±43 nmol $[Ca^{++}]i$ observed with a 5-min incubation. This increase in $[Ca^{++}]i$ was not statistically significant. sCT (1 μM) significantly reduced the KCl-stimulated rise in intracellular calcium (458 versus 333 nmol, respectively).

In synaptosomes incubated for 1 h prior to the addition of morphine (1 μM), the morphine attenuated the stimulated rise in $[Ca^{++}]i$ by 43% (Fig. 3). This effect of morphine was reversed by naloxone (1 μM) and was stereoselective in that (+) morphine (10 μM) did not alter KCl-induced rises in intracellular calcium (data not shown). When sCT (1 μM) was incubated with the synaptosomes for 1 h prior to the addition of morphine (1 μM), the combined attenuation of stimulated rises in $[Ca^{++}]i$ was 53% (Fig. 3). A similar effect was observed when synaptosomes were incubated for 5 min with sCT prior to morphine (1 μM) (data not shown). With either preincubation time, the sCT did not significantly alter the attenuation of depolarization-induced rises in intracellular calcium induced by morphine.

Effects of sCT on the Electrically Stimulated GPI

In the electrically stimulated GPI bioassay (Fig. 4), sCT was added to the 10-ml bath to yield final concentrations of 10^{-8} M through 10^{-5} M. Salmon CT (10^{-8} M) was inactive; 6.25×10^{-7} M sCT was minimally active (inhibition <10%); and 1.9×10^{-6} M sCT did not produce any greater inhibition than that produced by 1.25×10^{-6} M sCT, as shown in Fig. 4. sCT produced an increased height of contraction when first added to the bath (Fig. 4). This phase, which lasted 1 min or less, was followed by inhibition (25%) of the contraction of the ileum, which was reversed by naloxone (10^{-7} M). When sCT ($1.25\times$

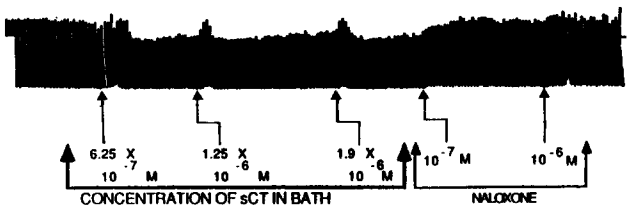

FIG. 4. Salmon-CT-induced modulation of the contracture of the electrically stimulated guinea pig ileum. Strips of ileum were prepared as described in the Method section. Salmon CT, vehicle or naloxone was added to the bath. This figure depicts a representative experiment. Each experiment was repeated in three separate strips of ileum and on 3 separate days.

10^{-6} M) was added to the bath 5 min prior to morphine, the morphine dose-response curve was shifted in a nonparallel fashion to the right (Fig. 5) as determined by the method of Tallarida and Murray (56). When sCT (1.25×10^{-6} M) was incubated with the ileum for 2 h prior to morphine, the morphine dose-response curve was not significantly shifted. In the nonstimulated GPI bioassay (data not shown), basically a cholinergic assay, sCT (10^{-8} M through 10^{-5} M) produced a contracture of the ileum (30% of the maximal effect observed with acetylcholine, 10^{-4} M) which was not dose related, did not occur in the absence of Ca^{++} in the bath, and was blocked by atropine (10^{-6} M). Therefore, sCT probably did not induce contracture of the nonstimulated ileum by causing the release of intracellular Ca^{++}, but rather by increasing uptake of Ca^{++} from extracellular sources, thus facilitating the cholinergic response which was blocked by atropine. sCT may cause increases in Ca^{++} uptake to the electrically stimulated ileum as well, thus increasing contraction height. Pretreatment of the ileum (Fig. 5) with

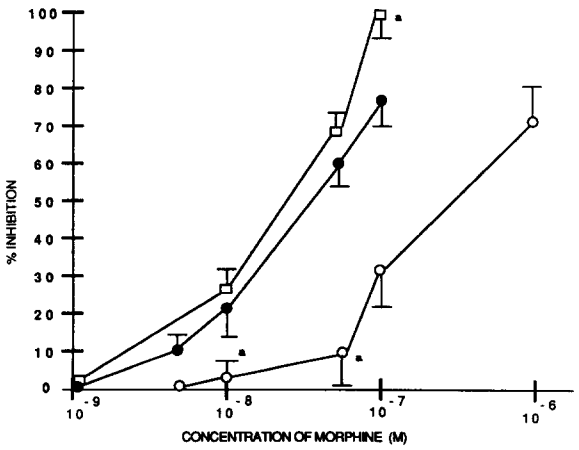

FIG. 5. Salmon-CT-induced modulation of morphine in the electrically stimulated guinea pig ileum. Strips of ileum were preincubated with sCT (1 μM) or vehicle for 5 min or 2 h prior to the addition of morphine to the bath. The dose-response curve of the morphine was determined in the presence and the absence of sCT. Each point represents the mean and the standard error for each concentration of morphine for n = 5 separate experiments. ● Vehicle plus morphine—The dose-response curve of the morphine was not different in the ileum incubated with the vehicle for either 5 or 120 min. These groups were therefore pooled such that n = 10 for this group. ○ sCT for 5 min prior to morphine. □ sCT for 120 min prior to morphine. (a) $p<0.05$ from appropriate vehicle control.

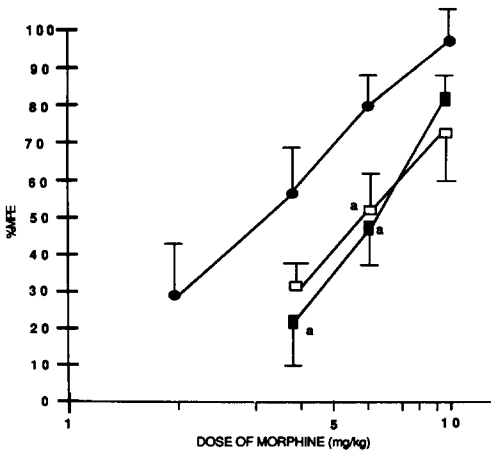

FIG. 6. Antagonism of morphine by sCT in the tail-flick test in mice. Mice were injected with distilled water vehicle, sCT (1.6 μg, ICV), or sCT (0.0016 μg, ICV) in combination with naloxone (0.01 mg/kg, SC), at 5 min prior to morphine. The dose-response curve of morphine was determined following the various pretreatments. Each pretreatment represents the mean and standard error for an n = 12–18 mice per dose. ● Vehicle plus morphine. ■ sCT (1.6 μg) at 5 min prior to morphine. □ sCT (0.0016 μg) plus naloxone (0.01 mg/kg) at 5 min prior to morphine. (a) $p<0.05$ from morphine/vehicle combination.

1.25×10^{-6} M sCT for 5 min attenuated morphine-induced inhibition of the electrically stimulated contraction. Although pretreatment of mice for 2 h with sCT slightly enhanced the activity of low doses of morphine in the tail-flick test (62), a similar enhancement in the activity of morphine in the GPI was not observed at this time point. Naloxone reversed sCT-induced antinociceptive effects in the tail-flick test, as well as sCT-induced inhibition of the stimulated GPI.

Effects of sCT on Morphine-Induced Antinociception

In the tail-flick test (Fig. 6), pretreatment of mice for 5 min with 1.6 μg sCT ICV shifted the ED_{50} of morphine from 3.3 mg/kg (CLS 2.0–5.4) to 5.7 mg/kg (CLS 2.9–11.5). Salmon CT (1.6 μg, ICV) significantly antagonized the antinociceptive activity of 4- and 6-mg/kg doses of morphine (Fig. 6). Salmon CT (0.0016 μg), inactive as an antagonist of morphine in the tail-flick test, facilitated the antagonism of morphine by a marginally active (<15% antagonism) dose of naloxone (0.01 mg/kg, SC) when the two drugs were administered concurrently 5 min prior to morphine in the tail-flick test (Fig. 6). Thus the antagonism of morphine (4 and 6 mg/kg) by sCT and naloxone in combination was significantly greater than that produced by naloxone (0.01 mg/kg) alone. All shifts in the morphine dose-response curve were essentially parallel, as determined by the method of Tallarida and Murray (56) (Fig. 6).

DISCUSSION

Modulation of [Ca^{++}]i by sCT and Morphine

The role of CT in central neuronal function has been demonstrated by many investigators. However, the mechanism by which CT produces central effects has not been clearly eluci dated. Ca^{++} modulation by sCT has been demonstrated in the hypothalamus (42) and in synaptosomes prepared from the hypothalamus (33). Salmon CT has also been shown to alter calmodulin-mediated protein phosphorylation, which could account in part for its alteration of calcium fluxes (21,47). No studies of

the effect of sCT on $[Ca^{++}]i$ have been previously reported. Our data clearly indicate that sCT produces alterations in $[Ca^{++}]i$ in whole-brain synaptosomes. It is not surprising that sCT increases basal levels of $[Ca^{++}]i$ in synaptosomes in light of the reported sCT-induced increases in Ca^{++} uptake peripherally to bone, bone cells in culture, kidney cells and chondrocytes (3, 28, 43, 44). Salmon CT has also been shown to enhance Ca^{++} conductance in neuronal membranes of the parasympathetic ganglia of the urinary bladder (45). The increases in depolarization-induced levels of intracellular calcium are similar to those observed using calcium uptake to the synaptosomes (64). The decreases in KCl-stimulated levels of $[Ca^{++}]i$ observed in synaptosomes with sCT (Table 1) are similar to the decreases in calcium uptake reported by Morley and Levine (42) and Koida et al. (33) in the hypothalamus and decreases in free intracellular calcium reported by Epand et al. (13) in pituitary cells. The mechanism by which sCT initially increases basal and KCl-stimulated $[Ca^{++}]i$ while decreasing KCl-stimulated levels of $[Ca^{++}]i$ at 1 h is yet to be elucidated and is currently under investigation in our laboratory. The increases in basal $[Ca^{++}]i$ are probably due to influx of calcium, since these increases are abolished by eliminating extracellular calcium. It is clear from these studies that the effects of sCT on basal intracellular calcium are small. This could be due to the use of whole brain, which may obscure smaller discrete changes in intracellular calcium that occur. However, the sCT-induced increases in stimulated $[Ca^{++}]i$ are robust and are probably due to influx of calcium via voltage-regulated "L-type" calcium channels, since these increases are attenuated by nifedipine, a calcium channel blocker. Since the administration of calcium ICV has been shown to attenuate morphine-induced antinociceptive effects (26), the rise in $[Ca^{++}]i$ produced by sCT probably results in sCT-induced blockade of morphine-induced antinociception in the tail-flick test and in the GPI.

The effect of morphine on KCl-stimulated levels of $[Ca^{++}]i$ is small but statistically significant. This could be due to the use of whole brain, or the fura-2 may be buffering small changes in intracellular calcium. Studies are ongoing using brain regions in order to address these problems. The effects of a 1-h pretreatment with sCT on morphine-induced alterations in intracellular calcium (Fig. 3) are not significant. These data correlate with the lack of modulation of the effects of morphine in the GPI by 1-h pretreatment with sCT and the small modulation of morphine-induced antinociception observed by 1-h pretreatment with sCT in vivo. Salmon CT does not appear to interact with the opiate receptor directly. One would expect that, if sCT were directly interacting with the opiate receptor, morphine-induced attenuation of stimulated rises in $[Ca^{++}]i$ might be potentiated by sCT. In addition, binding studies in our laboratory show no displacement of ^{3}H-naloxone binding to mouse or rat brain membranes by sCT (59), although we cannot rule out the possibility that naloxone alters sCT binding. Although naloxone did reverse the effects of sCT in some antinociceptive tests and in the guinea pig ileum bioassay, naloxone reversal is a necessary but insufficient criterion for classification of an effect as opiate-like. Thus the naloxone reversal of sCT effects in vivo and in vitro could indicate some opiate receptor interaction. It is more likely, however, in light of the synaptosomal data and binding data (61,63), that sCT and morphine have dissimilar mechanisms of action. The sCT, while not directly interacting with the opiate receptor, may have effects in opiate-sensitive pathways.

Correlation of Antinociception to Modulation of $[Ca^{++}]i$

Salmon CT has been shown to produce antinociceptive effects in a variety of species (including man) using a multitude of test systems (1, 4, 7, 14, 18, 49, 55, 62). Previous research reports indicate that sCT-induced antinociception becomes maximal between 1 and 2 h after administration, even when the sCT is injected directly into the periaqueductal gray matter (14) or lateral ventricle of the brain (4, 55, 62). Since the intact sCT structure is required for receptor binding (8, 16, 52, 54), and sCT does not rapidly degrade (14,54), production of active metabolites of sCT probably does not account for the lag time to the onset of an antinociceptive response. Since binding of sCT to its receptor is one-half maximal by 7 min at 37°C (16) and dissociation occurs very slowly (h) (14,16), binding kinetics probably do not account for the long onset of antinociceptive effects. Our data indicate that sCT (1 μM)-induced decreases in stimulated $[Ca^{++}]i$ at 1 h (Table 1) correlate temporally to the point at which sCT-induced antinociceptive responses are first observed. It is very difficult to extrapolate doses (ICV) in vivo to molar concentrations in vitro because the CSF volume in the mouse is very small [approximately 100 μl, as estimated from Fleming et al. (17)] and flow rates are not well established. However, injection of 1.6 μg/mouse ICV of sCT crudely approximates a concentration of 5×10^{-6} M sCT in the total mouse CSF at the moment of injection. sCT (1.6 μg, ICV) produces a maximal antinociceptive effect in mice at 2 h after administration (61), and its approximate corresponding concentration (1 μM) decreases stimulated levels of $[Ca^{++}]i$ at 1 h. Thus there appears to be a quantitative correlation between the concentration of sCT which decreases $[Ca^{++}]i$ in vitro and the dose of sCT which produces antinociception in vivo.

Salmon CT (1.6 μg, ICV) which antagonizes morphine-induced antinociception in the tail-flick test and morphine-induced inhibition of the contraction of the guinea pig ileum with 5 min pretreatment (Fig. 6), approximates the concentration (1 μM) of sCT that increases basal and KCl-stimulated $[Ca^{++}]i$ at 5 min. The sCT-induced increases in intracellular calcium could lead to the blockade of morphine's effects if 1) both morphine and sCT were interacting with a common pool of calcium, 2) the sCT-induced increase in $[Ca^{++}]i$ causes the release of another modulator which in turn blocks morphine, or 3) the sCT-induced increases in basal calcium could trigger the gating of channels, such as calcium and K^+ channels. The existence of such channel gating in synaptosomes has been documented (15,57). In addition, it has been hypothesized that morphine gates potassium channels by increases in intracellular calcium [as reviewed by Miranda and Paeile (41)]. The transient rise in basal calcium initially induced by sCT could be the trigger for a cascade of cellular events leading to the attenuation of depolarization-induced increases in $[Ca^{++}]i$ which occur upon a 1-h exposure to the peptide. Induction of such a cascade could explain the long onset of the sCT-induced antinociceptive effects observed in vivo (4, 14, 55, 61).

In summary, sCT-induced *decreases* in stimulated levels of $[Ca^{++}]i$ correlate well temporally to the antinociceptive effects of sCT which occur at 1 and 2 hours following administration. Salmon-CT-induced *increases* in basal and stimulated levels of $[Ca^{++}]i$ account for the blockade of morphine in the tail-flick test and in the electrically stimulated GPI which occur with 5 min pretreatment of mice or the GPI with sCT. Calcitonin may function in the brain as a neuromodulator by the regulation of central Ca^{++} fluxes. Due to the correlation between the sCT antinociceptive and modulatory effects in vivo and sCT-induced calcium modulation in synaptosomes (a model of presynaptic neuronal events), sCT modulation in the CNS may be largely presynaptic in location.

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