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THE ROLE OF BOUND CALCIUM IN SUPERSENSITIVITY INDUCED BY COCAINE

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1 Noradrenaline caused a small contraction of the cat isolated spleen strip bathed in a calcium-free solution; this contraction was greatly potentiated by cocaine. This potentiation was also present in isolated spleen strips where noradrenaline stores were depleted by reserpine. The maximum response of the spleen strip to noradrenaline in the absence of extracellular calcium was also increased by cocaine.

2 The disodium salt of ethylenediaminetetraacetic acid (Na-EDTA) but not Ca-EDTA antagonized the potentiation of the response to noradrenaline by cocaine in a calcium-free solution, and greatly reduced the magnitude of subsequent responses to noradrenaline and cocaine.

3 Strontium caused equivalent contractions of normal and reserpine-treated spleen strips bathed in a calcium-free solution. These responses were potentiated by cocaine.

4 Histamine caused a small contraction of the isolated spleen strip bathed in a Ca-free solution. Cocaine failed to potentiate these very small histamine contractions, but did potentiate the contraction of these same strips in response to noradrenaline.

5 It is concluded that the potentiation of the response of the isolated spleen strip to noradrenaline by cocaine in the absence of extracellular calcium is due to a mechanism other than decreased neuronal uptake of noradrenaline. It is suggested that cocaine makes a bound store of calcium more available to promote contraction of the spleen strip by noradrenaline.

Introduction

A number of recent studies suggest that the supersensitivity induced by cocaine is not entirely due to the blockade of neuronal uptake (Maxwell, Wastila & Eckhardt, 1966; Bevan & Verity, 1967; Kasuya & Goto, 1968; Reiffenstein, 1968; Kalsner & Nickerson, 1969; Varma & McCullough, 1969; Davidson & Innes, 1970; Innes & Karr, 1971; Shibata, Kuchii, Hattori & Fujiwara, 1974). Various other possible mechanisms have been considered. For example, increase of affinity of the receptors for the agonist (Maxwell *et al.*, 1966; Reiffenstein, 1968; Innes & Karr, 1971) has been proposed, but Innes & Mailhot (1973) showed that cocaine does not increase the affinity of α-adrenoceptors for noradrenaline. It has also been suggested that the supersensitivity induced by cocaine may be due to an alteration in the utilization of calcium for contraction (Greenberg & Innes, 1968; Kasuya & Goto, 1968; Greenberg & Long, 1971; Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971). We have therefore studied the effect of cocaine on the utilization of bound calcium in the contraction of cat spleen strips stimulated by noradrenaline.

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Methods

Cats (0.3–3 kg) of either sex were killed by a blow on the head. The spleen was removed and strips 20 mm long and 4–5 mm wide were cut from the edge. Each strip was suspended in a muscle bath containing 10 ml of bathing solution kept at 37°C, and bubbled with a gas mixture of 95% O_2 and 5% CO_2. Isotonic contractions against 1 g tension were recorded on a kymograph at ten times lever magnification. The strips were allowed to equilibrate in the bath for 1 h before drugs were added. The bathing fluid was replaced every 15 min except when drugs were present in the bath.

Bathing solutions were made with distilled demineralized water. Standard Krebs-Henseleit solution was of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose, 11.0. In experiments where the tissues were to be bathed in a medium without calcium, a 'Ca-free' solution was prepared by omission of $CaCl_2$ from the standard Krebs-Henseleit formula. To deplete the preparation of calcium the chelating agent disodium ethylenediaminetetraacetic acid (Na-EDTA), 0.3 mM, was added to the Ca-free solution; this was termed a 'zero-Ca EDTA' solution.

Stock solutions of all drugs were made in concentrations of 1, 5, or 10 mg/ml and stored at 4°C.

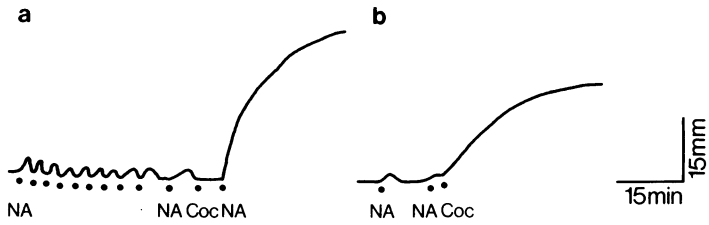

Figure 1 Potentiation of noradrenaline by cocaine in isolated spleen strip of the cat. Tissue calcium of two strips from the same spleen was reduced in Ca-free solution by repeated exposure to noradrenaline (NA, repeated at dots), 1 μg/ml, in a zero-Ca EDTA solution. (a) Cocaine (Coc), 10 μg/ml, was given 5 min before noradrenaline (NA), 1 μg/ml. (b) Cocaine (Coc), 10 μg/ml was given at the peak of the response to noradrenaline (NA), 1 μg/ml.

(–)-Noradrenaline bitartrate and tyramine hydrochloride were dissolved in 0.01 N HCl. Cocaine hydrochloride, EDTA disodium and dicalcium salts, strontium chloride, and calcium chloride were dissolved in distilled demineralized water. A stock solution of reserpine, 5 mg/ml, was made by dissolving 100 mg reserpine in 2 ml glacial acetic acid, 2.5 ml propylene glycol, 2.5 ml 95% ethanol, and sufficient water to make 20 ml. Cats were given 1 mg/kg reserpine intraperitoneally 24 h before an experiment. Spleen strips were shown to be depleted of noradrenaline by the loss of response to tyramine, 30 μg/ml. All drugs were appropriately diluted with 0.9% w/v NaCl solution (saline) on the day of use; with the catecholamines, 0.01 N HCl was added to delay oxidation. Drug concentrations are expressed as final concentration in the muscle bath in μg/ml, except calcium and strontium which are expressed in molar concentrations. Doses of noradrenaline and tyramine are expressed as free base, all other drugs as the salt.

Contractions of the spleen strip were measured at the peak response after they had reached a steady state. Relaxation was calculated as the difference between the magnitude of the peak contraction in response to an agonist and the contraction remaining after 30 min of exposure to an antagonist. When comparisons were made between spleen strips from the same cat, or within the same spleen strip the results were analysed by the *t* test for paired observations (Goldstein, 1964). Observations from different spleens were compared by Student's *t* test.

Results

Potentiation of noradrenaline by cocaine in a calcium-free solution

In these and subsequent experiments a Ca-free solution was used to eliminate an extracellular source of calcium, and zero-Ca EDTA solution was used to promote the removal of interstitial or loosely bound calcium.

Cocaine (10 μg/ml) potentiated the response to noradrenaline (1 μg/ml) in Ca-free solution. Spleen strips were first equilibrated in a zero-Ca EDTA solution for 60 minutes. Tissue calcium was then reduced by repeated additions of noradrenaline until the contraction obtained was small. The tissues were then bathed in Ca-free solution for 30 min to remove the EDTA. Noradrenaline was again added to the bath; a contraction was obtained; the bath was washed out and the tissue was allowed to relax. The strip was then either treated with cocaine for 5 min, after which noradrenaline was added to the bath in the presence of cocaine (Figure 1a), or cocaine was added after the response to noradrenaline had reached a plateau (Figure 1b).

In 36 strips from 32 cats pretreatment with cocaine significantly increased the height of the noradrenaline contraction by 9.9 ± 1.3 mm. In another 47 strips from 31 cats the superaddition of cocaine significantly increased the height of the noradrenaline contraction by 9.1 ± 1.2 mm. Many of these strips received further treatment, which is described in subsequent experiments.

Potentiation of the maximum contraction to noradrenaline by cocaine in calcium-free solution

Experiments were done on 8 strips, two from each of 4 cats. Strips were first bathed in a zero-Ca EDTA solution for 2 h to remove tissue calcium. The bathing solution was then changed to a Ca-free solution to remove the EDTA. Cumulative doses of noradrenaline (1 to 300 μg/ml) were added to the bath until a maximum contraction was obtained (Figure 2). Cocaine (10 μg/ml) was added to the bath when noradrenaline caused no further contraction. Without cocaine the maximum height of the noradrenaline contraction was 21.8 ± 5.4 mm, reached with concentrations of 30 or 100 μg/ml. Cocaine significantly

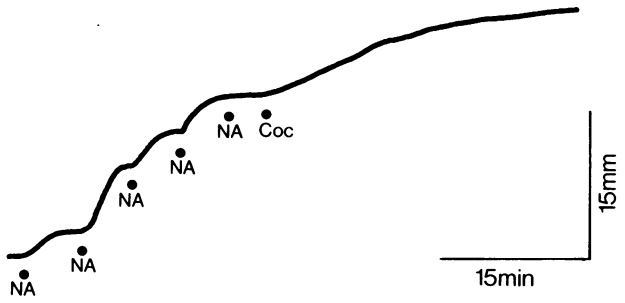

Figure 2 Potentiation of the maximum contraction to noradrenaline by cocaine in isolated spleen strip of cat in Ca-free solution. Strip was kept in zero-Ca EDTA solution for 2 h, then tested in Ca-free solution with noradrenaline (NA) in cumulative concentrations of 1, 3, 10, 30, 100 μg/ml, followed by cocaine (Coc), 10 μg/ml.

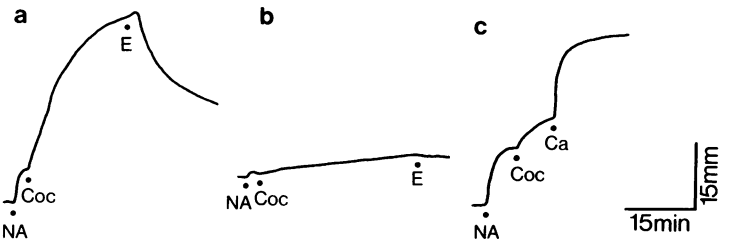

Figure 3 The effect of repeated administration of Na-EDTA on the potentiation of noradrenaline by cocaine in a single isolated spleen strip of cat in Ca-free solution. At NA, noradrenaline 1 μg/ml, at Coc, cocaine 10 μg/ml, at E, Na-EDTA 1 mg/ml and at Ca, calcium 2.5 mM. Between (a) and (b) the strip relaxed for 30 minutes. Between (b) and (c) the strip was kept in Krebs-Henseleit solution for 60 min, which was replaced by Ca-free solution just before the test with noradrenaline.

increased the height of the maximum noradrenaline contraction by 3.5 ± 1.1 mm ($P < 0.02$).

The effect of Na-EDTA on the potentiation of noradrenaline by cocaine in a calcium-free solution

The above results indicate that cocaine may enable noradrenaline to utilize membrane or intracellular bound calcium more effectively. Experiments were therefore done to see if Na-EDTA would alter the potentiation of noradrenaline by cocaine in a Ca-free solution, possibly by chelating this calcium once it was released from its binding site.

Eight spleen strips each from a different cat were first bathed in a zero-Ca EDTA solution for 2 h to reduce tissue calcium. The bathing solution was then changed to Ca-free and kept in this solution for 30 min to remove the EDTA. Noradrenaline (1 μg/ml) caused a small contraction of 6.8 ± 1.0 mm. The addition of cocaine (10 μg/ml) to the bath significantly increased the height of this contraction by 20.6 ± 4.0 mm. The further addition of Na-EDTA (1 mg/ml) caused the strips to relax by 15.7 ± 2.8 mm, thus reducing the potentiation caused by cocaine (Figure 3a). After 30 min in Ca-free solution noradrenaline, cocaine and Na-EDTA were tested again and the noradrenaline contraction, the potentiation by cocaine, and the relaxation by Na-EDTA were all reduced, being 2.8 ± 0.9, 2.1 ± 0.4, and 1.1 ± 0.3 mm respectively (Figure 3b). The preparation was then bathed in standard Krebs-Henseleit solution to replace some of the tissue calcium. After 1 h the bathing solution was changed back to Ca-free. After this replacement of calcium the same dose of noradrenaline caused a larger contraction, 13.8 ± 3.5 mm; this was increased by 7.9 ± 3.0 mm by cocaine (Figure 3c). The addition of 2.5 mM calcium caused a further contraction of 10.6 ± 2.1 mm.

The effect of Ca-EDTA on the potentiation of noradrenaline by cocaine in a Ca-free solution

To exclude the possibility that the inhibition of the cocaine potentiation of noradrenaline by EDTA might be a direct effect of EDTA rather than by chelation of calcium, four experiments were done, each on 2 strips

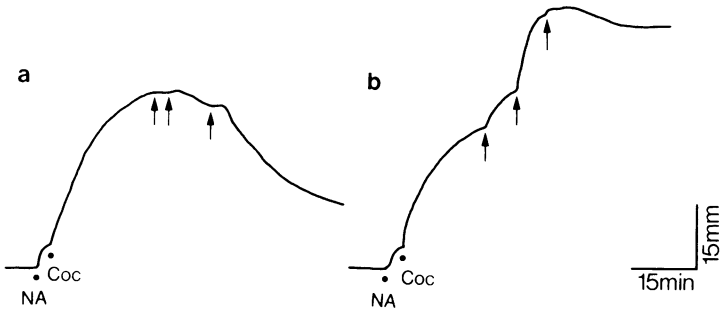

Figure 4 The effect of Na-EDTA and Ca-EDTA on the potentiation of noradrenaline by cocaine in isolated spleen strip of cat in Ca-free solution. Two strips from the same spleen were exposed to noradrenaline (NA), 1 μg/ml, followed by cocaine (Coc), 10 μg/ml, and then exposed at arrows to either Na-EDTA or Ca-EDTA in cumulative concentrations of 10, 100, 1000 μg/ml. (a) Treated with Na-EDTA; (b) treated with Ca-EDTA.

cut from the same spleen. One strip was tested with Na-EDTA and the other with Ca-EDTA (Figure 4). Both strips were first bathed in a zero-Ca EDTA solution for 2 h to reduce the tissue calcium. The bathing solution was then changed to Ca-free, to remove the Na-EDTA. Noradrenaline (1 μg/ml) caused a small contraction which was potentiated by the addition of cocaine (10 μg/ml) to the bath. The strips were then exposed to either Ca-EDTA or Na-EDTA in cumulative concentrations of 10 μg, 100 μg, and 1 mg/ml. All three concentrations of Ca-EDTA substantially increased the already potentiated contraction by 6.5 ± 1.7, 20.8 ± 3.4, 28.0 ± 3.4 mm respectively. The first two concentrations of Na-EDTA did not change the noradrenaline cocaine contraction, but the largest concentration substantially relaxed the strip by 20.0 ± 6.4 mm.

Four experiments were done on strips with normal Krebs-Henseleit solution. Ca-EDTA or Na-EDTA (1 mg/ml) alone had no effect, but Na-EDTA significantly reduced the contraction to noradrenaline (1 μg/ml) by 3.5 ± 0.9 mm ($P < 0.05$) whereas Ca-EDTA had no effect.

Spleen strips taken from four cats pretreated with reserpine, 1 mg/kg, were bathed in a Ca-free solution. Cocaine, 10 μg/ml, potentiated the response to noradrenaline 1 and 10 μg/ml. This potentiation was inhibited by Na-EDTA, 1 mg/ml.

Potentiation of the strontium-induced contraction by cocaine in a calcium-free solution

Strontium has been shown to cause contraction of smooth muscle (Daniel, 1963) and can substitute for calcium in the contraction of smooth muscle (Daniel, 1965; Hudgins, 1969; Toda, 1972). Experiments were done to see if strontium would contract the isolated spleen strip in the absence of extracellular calcium, and whether cocaine would potentiate this response.

Eight spleen strips from 4 normal cats and 8 spleen strips from 4 cats given reserpine (1 mg/kg, 24 h previously) were used; each strip served as its own control. Calcium was removed by exposure of the normal strips to noradrenaline (1 μg/ml) in the presence of a zero-Ca EDTA solution, and by bathing the reserpine-treated tissues in a zero-Ca EDTA solution for 2 hours. All strips were then kept in Ca-free solution. The strips from reserpine-treated cats were first tested with tyramine (30 μg/ml) for completeness of catecholamine depletion; no contraction occurred. Strontium added to these strips caused contraction. Four normal and four reserpine-treated strips were exposed to cumulative concentrations of 0.16 to 12.0 mM strontium until the maximum contraction was obtained. These strips were then re-exposed to the same strontium concentrations in the presence of cocaine (10 μg/ml). In four additional reserpine-treated and 4 normal strips from the same cats the order of treatment was reversed; the tissues were treated with cocaine first followed by the control test of strontium 150 min after the cocaine had been removed. Strontium caused a maximum contraction of 9.1 ± 3.3 and 7.5 ± 1.5 mm in the normal and reserpine-treated strips respectively. In the presence of cocaine the contraction of the same spleen strips was 29.0 ± 4.7 and 15.5 ± 1.9 mm respectively.

Potentiation of histamine by cocaine in a calcium-free solution

Experiments were done with 16 strips from 8 cats. The strips were first equilibrated in a zero-Ca EDTA solution for 1 hour. Tissue calcium was then reduced by repeated addition of histamine to the bath until a contraction of no more than 1 or 2 mm was obtained. The tissues were then bathed in a Ca-free solution to remove the EDTA. Histamine (1 μg/ml) was then

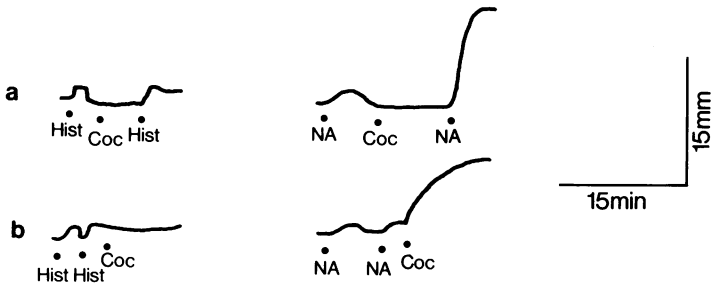

Figure 5 The effect of cocaine on the histamine-induced contraction of the isolated spleen strip of cat in Ca-free solution; strips (a) and (b) exposed to histamine (Hist), 1 μg/ml, before and in the presence of cocaine (Coc), 30 μg/ml; 150 min later both strips (a) and (b) exposed to noradrenaline (NA), 1 μg/ml before and in the presence of cocaine (Coc), 30 μg/ml.

tested in four strips 5 min after cocaine (30 μg/ml) had been added. In another 12 strips cocaine (30 μg/ml) was added after the contraction due to histamine (1 μg/ml) had reached its maximum. Cocaine did not potentiate the contraction in either case (Figure 5). However, 150 min later cocaine was still able to potentiate the response to noradrenaline (1 μg/ml).

Discussion

The results suggest that potentiation of noradrenaline by cocaine in spleen strips in the absence of calcium is probably due to a mechanism other than decreased neuronal uptake of noradrenaline. Although other possibilities cannot be excluded, the most likely explanation is that cocaine in some way makes a bound store of calcium more available for contraction.

Maengwyn-Davies & Koppanyi (1966) found that large concentrations of cocaine (1 mg/ml) caused the contraction of the rabbit isolated aortic strip by the release of endogenous catecholamines. Daniel & Wolowyk (1966) showed that very large concentrations of cocaine caused the contraction of the isolated uterus, by a direct action on the smooth muscle. However, in our experiments, where the maximum concentration of cocaine used was 10 μg/ml, much less than in the above experiments on aortic strip and uterus, cocaine never caused contraction of the spleen strip bathed in a calcium-free solution, but did potentiate the small contractions caused by noradrenaline. Depletion of the catecholamine stores by reserpine did not affect this potentiation by cocaine. Kirpekar & Wakade (1968) showed that extracellular calcium is necessary for the release of noradrenaline from postganglionic sympathetic nerves in the cat spleen. Therefore neither a direct contractile effect of cocaine on the smooth muscle nor release of endogenous catecholamines appears to be responsible for the potentiation by cocaine in the absence of extracellular calcium.

Our finding that cocaine increased the maximum response to noradrenaline in the spleen strip bathed in a calcium-free solution agrees with observations by Barnett, Greenhouse & Taber (1968) and Kasuya & Goto (1968) on rat isolated vas deferens in low calcium solutions. The increased maximum contraction is unlikely to be due to a greater noradrenaline concentration at the receptor as the result of blockade of neuronal uptake by cocaine, because the maximum contraction was obtained with noradrenaline concentrations of 30 μg/ml and 100 μg/ml and further increases in the concentration of noradrenaline did not cause additional contraction, but cocaine did.

Cocaine potentiated the responses to noradrenaline in a calcium-free solution, which were inhibited by EDTA. These results could be accounted for by a more effective release of bound calcium by noradrenaline, or by a more efficient use of the amount of calcium released by noradrenaline. These results are in contrast to the findings of Shibata *et al.* (1971) who found that cocaine did not potentiate the contraction of the rabbit aorta in response to noradrenaline in a Ca-free solution. The reason for this discrepancy might be explained by tissue and species differences, or by the possibility that loosely bound calcium was not removed in Shibata's experiments.

The experiments with strontium provide further evidence for a post-junctional site of action for cocaine. Strontium can substitute for calcium in the contraction of smooth muscle (Sperelakis, 1962; Daniel, 1963; Bohr, 1964; Hudgins, 1969), possibly by displacing bound intracellular calcium (Daniel, 1965; Toda, 1972). Our experiments where pretreatment of the cat with reserpine did not reduce the contraction caused by strontium in spleen strips bathed in calcium-free solution indicate that the contraction was not due to the release of stored noradrenaline. Our results are fully consistent with the hypothesis that strontium causes contraction of the isolated spleen strip by releasing bound calcium, and

that this bound calcium is much more easily released in the presence of cocaine. The results therefore suggest a post-junctional and perhaps identical site of action for the effect of cocaine on the contractions due to both noradrenaline and strontium in the calcium-depleted tissue.

Spleen strip contractions due to histamine were greatly reduced by the absence of extracellular calcium, and by loss of tissue calcium from repeated exposure to histamine in the presence of a zero-Ca EDTA solution. Cocaine failed to potentiate these very small histamine contractions, but did potentiate the contraction of these same strips in response to noradrenaline. These results are in agreement with the findings of Hudgins & Weiss (1968) who showed that histamine depends mainly on extracellular and loosely bound calcium for the contraction of smooth muscle, while noradrenaline can utilize a tightly bound calcium store. It is possible that cocaine makes a bound calcium store more available for contraction, and that this calcium is then utilized by noradrenaline and not by histamine.

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