Br. J. Pharmac. (1974), **51**, 577-583

EFFECT OF 6-HYDROXYDOPAMINE ON [^{3}H]-ADENINE NUCLEOTIDE AND [^{3}H]-NORADRENALINE RELEASE FROM THE GUINEA-PIG TAENIA CAECUM EVOKED BY ELECTRICAL FIELD STIMULATION, NICOTINE AND PERIVASCULAR NERVE STIMULATION

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1 The effects of pretreatment of guinea-pigs with 6-hydroxydopamine (6-OHDA) on the release of [^{3}H]-noradrenaline and [^{3}H]-adenine nucleotide following electrical field, nicotine and perivascular nerve stimulation of guinea-pig taenia caecum were studied.

2 High frequency electrical field stimulation (15,30 Hz) released both [^{3}H]-noradrenaline and [^{3}H]-adenine nucleotide but low frequency (0.5, 5 Hz) stimulation, producing comparable muscle relaxation led only to [^{3}H]-noradrenaline release.

3 6-OHDA (50-200 mg/kg) pretreatment inhibited the muscle relaxation and [^{3}H]-noradrenaline release with electrical stimulation or with nicotine in isolated taenia but did not affect the release of [^{3}H]-nucleotide.

4 A low dose of 6-OHDA (50 mg/kg), completely inhibited the muscle relaxation and [^{3}H]-noradrenaline release elicited by perivascular nerve stimulation.

5 Both tissue noradrenaline content and [^{3}H]-noradrenaline uptake were decreased to the same extent by low as well as high doses of 6-OHDA: noradrenaline content was reduced to 20% and uptake to 30% of the control value.

6 Catecholamine fluorescence disappeared from tissue layers of the taenia after treatment with a high dose of 6-OHDA.

7 In these experiments the inhibitory action of electrical stimulation and nicotine on the taenia can be correlated better with noradrenaline than with nucleotide release.

Introduction

Electrical field (transmural) stimulation and administration of nicotine produce similar relaxation of the guinea-pig taenia caecum. Blockade of the adrenergic system by various agents does not markedly affect the transmurally-evoked inhibition (Burnstock, Campbell & Rand, 1966; Bianchi, Beani, Frigo & Crema, 1968; Crema, Del Tacca, Frigo & Lecchini, 1968; Day & Warren, 1968), and Burnstock and co-workers (see Burnstock, 1972) have postulated the presence of non-adrenergic inhibitory neurones in addition to the adrenergic fibres in the mammalian intestinal tract. According to their hypothesis, the perivascular nerve pathway conveys the adrenergic influence, whereas the responses to transmural stimulation and to application of ganglion-stimulating agents such as nicotine and dimethylphenylpiperazinium (DMPP) are mediated through the intramural, non-adrenergic nerves. Moreover, since the intestinal smooth muscle preferentially accumulates adenine nucleoside and the relaxation in response to electrical stimulation was associated with the release of adenine nucleotide, these investigators proposed that the neurohumoral transmitter of the non-adrenergic neurone may be ATP or a closely related nucleotide (Burnstock, 1972). Hattori, Kurhashi, Mori & Shibata (1972) and Kuchii, Miyahara & Shibata (1973a, b) found in cold stored preparations that inhibition of the taenia caecum evoked by transmural stimulation, nicotine and DMPP, can still be attributed to stimulation of adrenergic nerves.

The present study was undertaken to compare

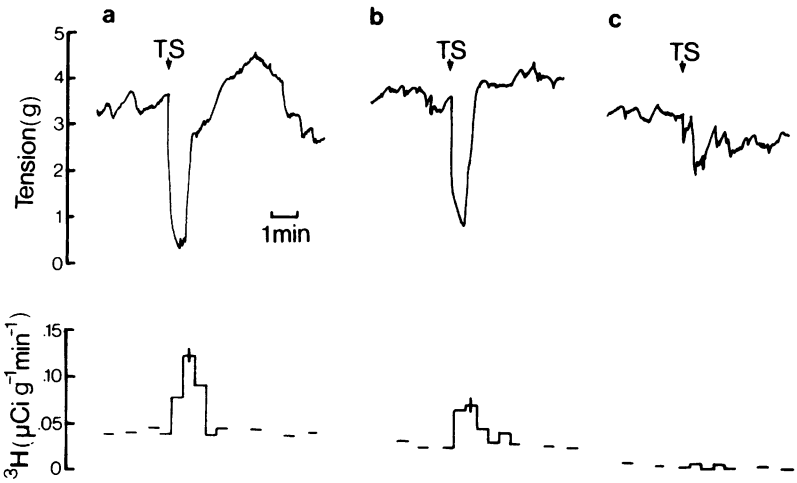

Fig. 1 Effect of 6-hydroxydopamine (6-OHDA) on the muscle tension changes and release of [^{3}H]-noradrenaline ([^{3}H]-NA) evoked by application of electrical field stimulation (at arrow). Upper traces show the mechanical responses and lower traces [^{3}H]-NA release for a control preparation (a) and after treatment with 6-OHDA (50 mg/kg) for 2 days in two different preparations (b and c).

the inhibitory effect of transmural stimulation, of perivascular nerve stimulation and of nicotine on the taenia caecum, paying particular attention to the release of [^{3}H]-noradrenaline ([^{3}H]-NA) and [^{3}H]-adenine nucleotide following chemical sympathectomy with 6-hydroxydopamine.

Methods

Isolated taenia caecum strips from guinea-pigs were prepared and set up as described by Kuchii *et al.* (1973a). For 6-hydroxydopamine (6-OHDA)-treated preparations the guinea-pigs had been previously injected intraperitoneally with 50, 100 or 200 mg/kg of 6-OHDA (Regis Chemical Co.) on 2 consecutive days and used on the 3rd day.

In the transmurally stimulated preparation, the taenia strip was suspended between two parallel platinum wire electrodes; the tissue was superfused with Krebs solution at a rate of 2.2 ml/min through a Harvard compact infusion pump. Electrical stimuli were rectangular pulses of 0.15 ms duration at 60 V, delivered at 0.5-30 Hz for 30 s through a Grass model 5 stimulator. The perivascular nerve was stimulated supramaximally for 10 s with pulses of 1.5 ms duration at 60 Hz. Intervals between stimulation were at least 5 min to ensure complete recovery of the tissue.

Radioisotopic studies of noradrenaline and adenosine release were carried out as described by Kuchii *et al.* (1973a).

Results

Electrical stimulation

The taenia strips showed the characteristic biphasic response, relaxation followed by contraction, following transmural stimulation at frequencies from 0.5-30 Hz. With electrical stimulation, the amplitude of the inhibitory response was dependent on the frequency, as well as on the intensity of stimulation (Kuchii, *et al.*, 1973a). A maximum relaxation usually occurred at 15 Hz or at a lower frequency of stimulation for the varying intensities of stimulus employed. Relaxation produced by the high frequencies (15 and 30 Hz) of transmural stimulation was accompanied by marked release of [^{3}H]-adenine nucleotide from the taenia. With low frequency (0.5 and 5 Hz) stimulation the release of [^{3}H]-nucleotide was barely greater than the background control. In contrast, stimulation at high (30 Hz) or low (0.5 Hz) frequency caused the same level of [^{3}H]-NA release from the tissue; both stimuli increased [^{3}H]-NA release, by 0.65 ± 0.1 and 0.68 ± 0.08 μCi g^{-1} min^{-1} (s.e. mean, $n = 10$), respectively, over the background level.

In the taenia of guinea-pigs pretreated with 6-OHDA, the inhibitory effect of electrical stimulation was not consistent and varied in the different preparations. Figure 1a illustrates the control response to electrical stimulation on the

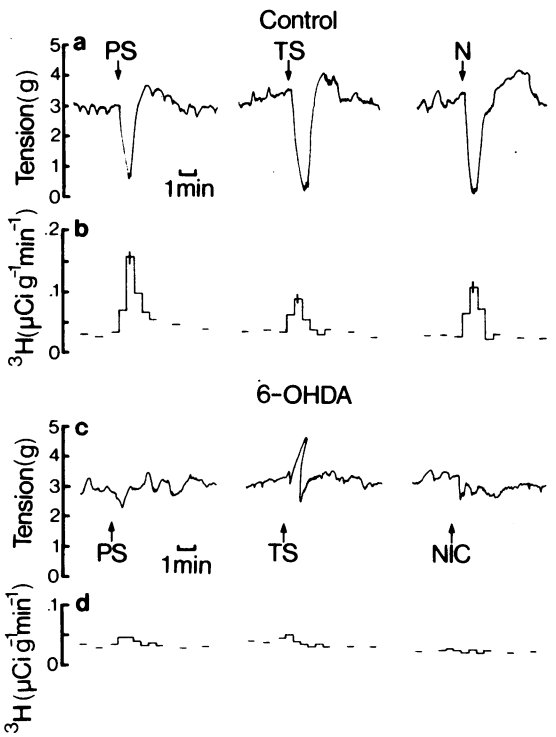

Fig. 2 Effect of a high dose of 6-hydroxydopamine (6-OHDA) on the muscle tension changes and release of [^{3}H]-noradrenaline ([^{3}H]-NA) evoked by perivascular nerve stimulation (PS, 1.5 ms, 50 V, 60 Hz for 10 s) electrical field stimulation (TS, 0.15 ms, 60 V, 30 Hz for 30 s), or nicotine (Nic, 10^{-4}M). a and b show representative responses from untreated taeniae (controls); c and d from 6-OHDA (i.p., 200 mg/kg for 2 days) treated preparations.

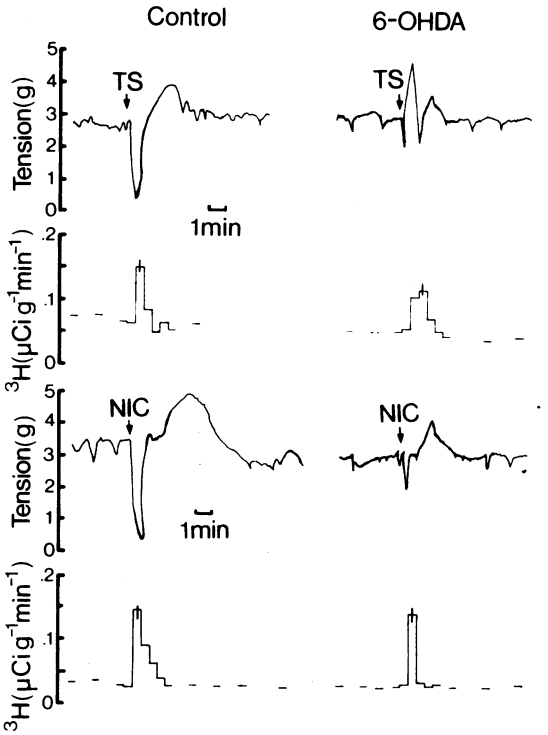

Fig. 3 Effect of a high dose of 6-hydroxydopamine (6-OHDA) on muscle tension changes and [^{3}H]-nucleotide ([^{3}H]-Nuc) release produced by electrical stimulation (TS) and nicotine (Nic). The records on the left illustrate responses obtained in untreated controls; the responses on the right were obtained from taeniae following treatment with 6-OHDA (200 mg/kg for 2 days).

relaxation and release of [^{3}H]-NA in untreated preparations. Treatment with a low dose (50 mg/kg) caused a reduction in the relaxation in most preparations but did not completely abolish the inhibitory response (Figure 1b). In these preparations, stimulation released detectable amounts of [^{3}H]-NA, but the level of release was significantly less than in the untreated preparations ($P < 0.01$ $n = 15$) (Figure 1a & b). In still other preparations (5 out of 20 observations) the 6-OHDA treatment almost abolished the inhibitory response but did not affect the excitatory component (Figure 1c). In these cases [^{3}H]-NA release was not observed.

Following pretreatment with high doses (100 mg/kg or 200 mg/kg) of 6-OHDA many of the preparations exhibited only a marked contraction as the inhibitory response to the electrical stimulation was completely blocked (Figure 2). In a few cases (3 out of 18), after such treatment, the taenia strip did show some, although reduced, relaxation. This residual relaxation response to the electrical stimulation was always accompanied by [^{3}H]-NA release. In marked contrast, tissues treated with low or high doses of 6-OHDA still released [^{3}H]-adenine nucleotide at a level comparable to that from the untreated controls (Figure 3).

Nicotine

In taenia strips pretreated with a low dose of 6-OHDA (50 mg/kg) the inhibitory action of nicotine (up to 10^{-4}M) was not consistent but varied from preparation to preparation as was the case with transmural stimulation. This variability was much less than that observed with electrical stimulation. In 5 of 18 6-OHDA-treated

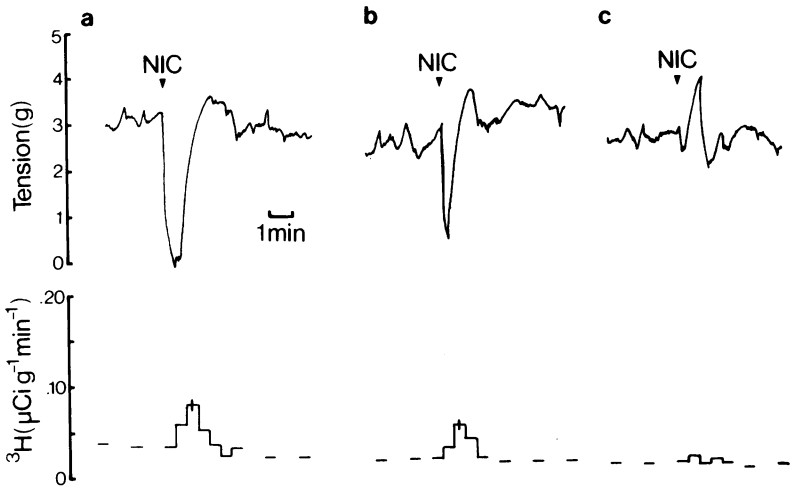

Fig. 4 Effect of 6-hydroxydopamine (6-OHDA) on muscle tension changes and [^{3}H]-noradrenaline ([^{3}H]-NA) release evoked by application of nicotine (Nic, 10^{-4}M). Upper traces represent the mechanical responses; the lower traces the release of [^{3}H]-NA from a control preparation (a) and following treatment with 6-OHDA (50 mg/kg) for 2 days in two different preparations (b and c).

preparations, nicotine still induced muscle relaxation and caused the release of [^{3}H]-noradrenaline, but both responses were significantly lower than those in the control preparations (Figure 4a & b). In other preparations, nicotine failed to evoke the inhibitory response and did not release [^{3}H]-noradrenaline, but caused the taenia strip to contract (Figure 4c). High doses of 6-OHDA (100 mg/kg or 200 mg/kg) in all preparations tested (15 experiments) blocked the relaxation and release of [^{3}H]-NA in response to nicotine but had no effect on the release of [^{3}H]-nucleotide (Figures 2 & 3).

Perivascular nerve stimulation

Previously it was shown that the relaxation evoked by stimulation of the perivascular nerves was associated with a proportionate increase in the release of [^{3}H]-NA (Kuchii *et al.*, 1973a). Although the release of [^{3}H]-adenine nucleotide was not easily detectable, a trace of [^{3}H]-adenine nucleotide always appeared in the superfusion fluid when the perivascular nerves were stimulated supramaximally.

In the present experiments, after treatment with 6-OHDA at a low dose (50 mg/kg), perivascular nerve stimulation failed to release [^{3}H]-NA and produced no relaxation in 20 preparations tested. The trace amount of [^{3}H]-adenine nucleotide release was not affected in these preparations.

In previous experiments, attempts were made to separate and identify the nucleotides released in the perfusion fluid following stimulation of the taenia strip. The results were at best inconclusive, presumably because of the low radioactivity in the separate fractions and because of the chemical alterations the released nucleotides undergo. Since the predominant biosynthetic product of the administered [^{3}H]-adenosine in taenia is ATP (Su, Bevan & Burnstock, 1971; Kuchii *et al.*, 1973b), it is assumed that ATP makes up the major part of the radioactive material released from the [^{3}H]-adenosine-treated tissues.

Uptake of ^{3}H-noradrenaline

Pretreatment with 6-OHDA significantly inhibited the NA uptake mechanism of the taenia. With the low dose of 6-OHDA (50 mg/kg) the uptake of [^{3}H]-NA was reduced by approximately 80%. In five experiments the tissue [^{3}H]-NA uptake (ct min^{-1} mg^{-1}) after 6-OHDA (50 mg/kg) was 777 ± 69 (s.e. mean), which was less than ($P < 0.001$) the control value, 3832 ± 27. Higher doses of 6-OHDA, did not enhance this inhibitory effect and the [^{3}H]-NA uptake did not differ from that observed with the low dose. The [^{3}H]-NA uptake with the high doses (100 mg/kg and 200 mg/kg) was 713 ± 56 and 700 ± 43, respectively.

Catecholamine content

6-OHDA had a similar effect on the tissue

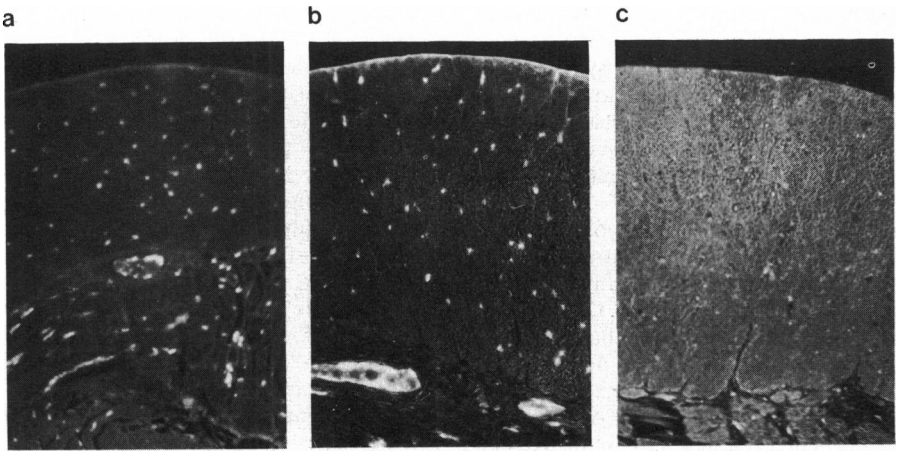

Fig. 5 Effects of 6-hydroxydopamine (6-OHDA) on specific fluorescence for tissue catecholamines. The micrographs illustrate the distribution and intensity of catecholamine fluorescence in the tissue layers of taeniae (cross section 50 x) without treatment (a), following 6-OHDA treatment (50 mg/kg for 2 days) in two different preparations (b and c).

noradrenaline content of the taenia caecum. At the three doses of 6-OHDA tested in five experiments the noradrenaline content was about 20% of the control value. The NA content before treatment was 1.36 ± 0.06 (s.e. mean) and after 6-OHDA (50 mg/kg) the NA level was reduced to 0.23 ± 0.04, which differed significantly ($P < 0.001$) from the control value. An increase in the dose of 6-OHDA did not cause much further depression of NA content; following the 6-OHDA (100 mg/kg) treatment the tissue NA content became 0.25 ± 0.06, whereas after 200 mg/kg it was 0.21 ± 0.04. These results were in accord with our previous results which indicated that reserpine (4 mg/kg, i.m., 24 h before the experiment) reduced tissue noradrenaline content to about 18% (Hattori *et al.*, 1972).

Histochemical study

The micrographs in Fig. 5 show the effect of 6-OHDA pretreatment on the specific fluorescence for catecholamines in the taenia caecum. A fluoroscopic cross section of an untreated guinea-pig taenia caecum shows the specific catecholamine fluorescence and the numerous structures with intense fluorescence (Figure 5a). In the taenia strips from guinea-pigs treated with a low dose (50 mg/kg) of 6-OHDA, in 8 of 20 observations the catecholamine fluorescence was still discernible in all tissue layers of taenia (Fig. 5b) as in the untreated preparations. These taenia strips were still capable of exhibiting relaxation and release of [^{3}H]-NA following transmural stimulation, and after nicotine, whereas other preparations which did not undergo relaxation and did not release [^{3}H]-NA were devoid of catecholamine fluorescence (Figure 5c). Following pretreatment with high doses (100 mg/kg and 200 mg/kg) of 6-OHDA, in all tests, the specific fluorescence disappeared from the tissue layers of the taenia.

Discussion

In our previous experiments we showed that although electrical stimulation of the taenia caecum is associated with efflux of both [^{3}H]-nucleotides and [^{3}H]-NA, only that for noradrenaline was proportional to the intensity of stimulation and relaxation (Kuchii *et al.*, 1973a). These data further demonstrated a dissociation of the inhibitory effect of nicotine and of transmural stimulation from [^{3}H]-nucleotide release in the cold stored as well as reserpine-treated taenia. Moreover, treatment with adrenoceptor blocking agents was shown to reduce markedly the relaxation produced by application of nicotine and transmural stimulation (Hattori *et al.*, 1972; Kuchii *et al.*, 1973b) and the inhibitory effect following stimulation of the perivascular nerve was accompanied by the release of NA but not adenine nucleotides (Kuchii *et al.*, 1973a).

In this paper further evidence is presented to show that the inhibitory action of transmural stimulation and of nicotine is mediated mainly through a mechanism other than the purinergic system. Whereas the relaxation elicited by intensive electrical stimulation at high frequency is

associated with the release of [^{3}H]-adenine nucleotides, the response at low frequency is not. In this regard the relaxation elicited by electrical stimulation can be correlated more closely to the release of NA, which resembles the relationship in the inhibitory response to perivascular nerve stimulation, where there is negligible [^{3}H]-nucleotide release.

Variation in tissue susceptibility to chemical sympathectomy by 6-OHDA has been reported by several investigators (Thoenen & Tranzer, 1968; Haeusler, Haefely & Thoenen, 1969; Goldman & Jacobwitz, 1971). It has been noted that 6-OHDA in low doses produces only a transient depletion of noradrenaline, but high doses produce a prolonged depletion of noradrenaline stores (Kostrzewa & Jacobwitz, 1972). The present results indicate that in many instances, a low dose of 6-OHDA failed to abolish the relaxation and [^{3}H]-NA release associated with application of nicotine and transmural stimulation. Only after treatment with high doses of 6-OHDA was the mechanical response and NA release to transmural stimulation and nicotine abolished or markedly diminished.

It has been suggested that the effectiveness of the chemical sympathectomy by 6-OHDA can be readily assessed by the fluorescence of the tissue 24 h after a single dose (Hellmann, Hertting & Peskar, 1971). In the present experiments, histochemical study indicates that after a low dose of 6-OHDA, the tissue fluorescence picture is not always distinguishable from that of the untreated controls, although at this time the chemical and radioisotopic assays reveal a significant reduction in noradrenaline content, as occurs with high doses of 6-OHDA treatment. In such tissues, nicotine and transmural stimulation still caused relaxation and release of [^{3}H]-NA, indicating that the residual noradrenaline level did not reflect the proportional amount of viable adrenergic fibres remaining in the tissue. This histochemical study suggests that treatment with low doses of 6-OHDA fails to effect degeneration of all adrenergic nerves and does not produce a complete sympathectomy. Presumably, some of the adrenergic fibres in the taenia are resistant to 6-OHDA action.

Treatment with high doses of 6-OHDA caused the catecholamine fluorescence to disappear completely from all tissue layers in the taenia and markedly blocked the relaxation to electrical and chemical stimulation. Thus, at high doses of 6-OHDA the histochemical picture was consistent with the physiological status; blockade of neurogenic relaxation associated with chemical degeneration of adrenergic neurones in the taenia. These results support the notion that the inhibitory effects of transmural stimulation and nicotine are conveyed mainly through adrenergic neurones, as suggested in previous papers (Hattori *et al.*, 1972; Kuchii *et al.*, 1973a, b). The dissociation of the inhibitory effect produced by nicotine and transmural stimulation from [^{3}H]-nucleotide release in the 6-OHDA-treated tissues does not support the adenine nucleotide transmitter hypothesis.

Recently, Weisenthal, Hug, Weisbrodt & Bass (1971) postulated that the inhibitory activity of both transmural and perivascular nerve stimulation is mediated by the adrenergic system. The relaxation with transmural stimulation is caused by liberation of noradrenaline from the nerve terminals making direct contact with smooth muscle cells, whereas the site of the inhibitory action following perivascular nerve stimulation is mainly the excitatory ganglion cells in Auerbach's plexus. In their scheme nicotine may activate the perivascular nerves to produce its effects. If this hypothesis is correct, there would be no need to invoke a non-adrenergic mechanism to explain any of the inhibitory response. The differential blockade of inhibition induced by transmural stimulation, by perivascular nerve stimulation and by nicotine must then be explained by a difference in susceptibility of the adrenergic fibres. On this basis the response to perivascular nerve stimulation is more susceptible to cold storage, reserpine, 6-OHDA and adrenoceptor blocking agents than that evoked by transmural stimulation (Kuchii *et al.*, 1973a). Although this is an interesting hypothesis and can explain much of the mechanism underlying inhibitory activity in mammalian smooth muscle, it still cannot account for the non-adrenergic or purinergic inhibition observed by Burnstock *et al.* (1966).

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(Received February 15, 1974)