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SINGLE PULSE STIMULATION OF GUINEA-PIG VAS DEFERENS AND THE PRESYNAPTIC RECEPTOR HYPOTHESIS

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1 The effect of phenoxybenzamine on the efflux of [^{3}H]-noradrenaline and the mechanical response to single pulse excitation of superfused guinea-pig vas deferens was determined to examine the validity of the currently accepted hypothesis of a presynaptic negative feedback system on adrenergic nerve terminals.

2 The adrenoceptor antagonist enhanced both the outflow of tritium and the mechanical response to single pulse stimulation. The efflux of labelled material and the responses to 4 pulses were also enhanced, as expected.

3 Blockade of neuronal and extraneuronal uptake did not by itself increase nerve-induced outflow or the mechanical response nor did it prevent phenoxybenzamine from doing so.

4 The present observations cannot be accommodated within the framework of a hypothesis that proposes that the enhancement of response and tritium efflux by phenoxybenzamine results from blockade of a feedback system whereby noradrenaline released by previous impulses inhibits its own subsequent release.

Introduction

The hypothesis that adrenergic nerve terminals possess inhibitory receptors for noradrenaline which function as an integral part of a negative feedback system regulating neurotransmitter release rests on two fundamental sets of experimental observations: (1) exogenous noradrenaline and certain other sympathomimetics inhibit the efflux of [^{3}H]-noradrenaline from electrically stimulated tissues and (2) phenoxybenzamine, and to a lesser extent certain other adrenoceptor antagonists, increase the outflow of [^{3}H]-noradrenaline in neurally excited tissues. These findings have been interpreted as reflecting the activation and the blockade of a presynaptic receptor system, respectively. One of the most critical tests which can be applied to this hypothesis is to assess the effects of phenoxybenzamine on response magnitude and [^{3}H]-noradrenaline efflux after single pulse excitation of a sympathetically innervated tissue. Since noradrenaline released by a single pulse cannot retroactively affect its own release, such efflux must be immune from the effects of presynaptic receptor blockade.

It is appropriate that the tissue selected for the present experiments be the guinea-pig vas deferens as the presynaptic receptor hypothesis, in its modern form, can be traced to Hotta (1969) who observed that contractions of this preparation to nerve stimulation were reduced by exogenous noradrenaline. The vas has subsequently been routinely used to verify and expand the implications of the adrenergic presynaptic receptor system. The findings described here, that both the mechanical response and the efflux of [^{3}H]-noradrenaline are significantly increased by phenoxybenzamine under conditions of single pulse excitation, challenges seriously the validity of a hypothesis which attempts by means of a unitary formulation of an inhibitory feedback system, to explain certain actions of both agonists and antagonists on transmitter release and on the mechanical response.

Methods

Tissue preparation

Vasa deferentia of albino guinea-pigs (500 to 900 g) were dissected out, immersed in warmed (37°C) and oxygenated (95% O_2 and 5% CO_2) Krebs–Henseleit (Krebs) solution with added disodium edetate (0.01 g/ml) and promptly desheathed. Both vasa from a given animal were routinely incubated for 60 min in 4.0 ml of Krebs solution containing [1-7-^{3}H]-noradrenaline (10 μCi/ml, 11.0 μg/ml; 6.7×10^{-7} M). The tissues were then dipped in fresh Krebs solution, suspended between platinum wire electrodes and maintained under 1 g tension. Mechanical responses were recorded isometrically by force-displacement transducers and a Grass polygraph. The preparations

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were superfused continuously with oxygenated Krebs solution at 37°C by means of a gravity-feed apparatus at a constant pressure of 60 mmHg and the flow rate adjusted to 5 ml/min.

Intramural nerve stimulation was performed with one or four biphasic pulses (5 Hz) of 1.0 ms duration and at supramaximal voltage, using a model S5 Grass stimulator. Since these stimulators lack the necessary precision to deliver reproducibly one or four pulses to the tissues, they were driven by an external timer and frequency modulator. The frequency of this device was set to exactly five cycles per second by means of a cathode ray oscilloscope (CRO), and the 0.1 to 1.1 second timer was calibrated to permit only one or four pulses to reach the vas. As a further safeguard against erroneous pulse trains prejudicing experimental results, each tissue stimulation was monitored by the CRO and the actual number of pulses delivered was noted.

Drugs and radiochemicals

The drugs used and their sources were: cocaine hydrochloride (May & Baker Ltd.), hydrocortisone (Sigma), phenoxybenzamine hydrochloride (Smith Kline & French Canada Ltd.). The radioisotope, [1-7-^{3}H]-noradrenaline hydrochloride (sp. act. 15 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. It was diluted to a stock concentration of 100 μCi/ml (6.7×10^{-6} M) in ascorbic acid (50 μg/ml) and stored at 4°C in 10 ml aliquots under nitrogen gas. To obtain a final concentration of 10 μCi/ml (6.7×10^{-7} M) in the incubation medium, 0.4 ml of this stock solution was added to 3.6 ml of Krebs solution. The cocaine (3 μg/ml; 8.8×10^{-6} M) and phenoxybenzamine (10 μg/ml; 3.3×10^{-5} M) were directly incorporated into the Krebs solution. Hydrocortisone (10 μg/ml; 2.8×10^{-5} M) was initially dissolved in ethanol before addition to the Krebs solution.

To confirm viability of the preparations and before proceeding with the experiment, each vas received a primer set of stimuli consisting of two trains of three pulses (5 Hz) with a 2 min interval between them. After 90-min equilibration, two single pulses, 9 min apart, followed after 9 min by two sets of four pulses (5 Hz), 9 min apart, were applied to each vas. To determine the effects of phenoxybenzamine on tissue mechanical responses and efflux of [^{3}H]-noradrenaline, the haloalkylamine (3.3×10^{-5} M) was added to the superfusate reservoir after the completion of the control set of stimuli described above. Thirty minutes after the initial exposure to the antagonist, and in its presence, a second identical set of stimulations was performed. In those experiments where hydrocortisone and cocaine were used, the agents were present throughout the experiment having been added directly to the Krebs reservoir.

Efflux of [^{3}H]-noradrenaline

The basal and stimulation-induced efflux of [^{3}H]-noradrenaline from the tissues was determined by assaying 1.0 ml aliquots of the 5.0 ml superfusate collected in vials by a fraction collector which rotated at 1-min intervals. The aliquots were subsequently transferred to vials containing 10 ml of aqueous counting scintillant (Amersham) and counted to a 1% error in a Beckman LS-230 system with automatic external standardization to determine efficiency.

Stimulation-evoked efflux is expressed as disintegrations per minute (d/min) and was determined by subtracting the basal efflux, calculated as the mean of the two 1-min samples collected immediately before stimulation, from the stimulation-evoked efflux. Transmural stimulation, when given, was always initiated at the onset of a 1-min collection period.

All data are presented as means with their standard errors and differences were compared by Student's paired t test, unless otherwise indicated.

Results

Efflux of [^{3}H]-noradrenaline and responses to stimulation

The guinea-pig vas deferens responds to single pulse excitation with a distinct mechanical response and an efflux of tritiated noradrenaline raised significantly above basal levels. The mean contraction and stimulation-induced efflux in initial runs with 12 tests on 6 control preparations, after delivery of a single pulse, was 0.3 ± 0.1 g and $0.6 \times 10^3 \pm 0.2$ d/min (Figure 1). Stimulation with four pulses (5 Hz) yielded considerably greater effects, with means of 2.1 ± 0.3 g and $3.9 \times 10^3 \pm 0.4$ d/min. The stimulation-induced effluxes of tritium did not differ significantly from initial values when, after a 30-min interval, the cycle of two single pulses, 9 min apart, followed by two sets of four pulses, 9 min apart, was repeated on the same control test preparations (Figure 1). Mechanical responses also did not differ significantly between first and second runs when single pulses were administered but were slightly increased on the second run with four pulses.

In those preparations exposed to phenoxybenzamine (3.3×10^{-5} M) during the 30-min interval, after the initial control sets of single and multiple pulses, repetition of the stimulation cycle yielded significantly greater effects in all test parameters, as shown in Figure 1. In 28 tests on 14 vas the [^{3}H]-noradrenaline outflow in response to a single pulse delivered in the presence of phenoxybenzamine was 328% of that obtained before exposure to the adrenoceptor antag-

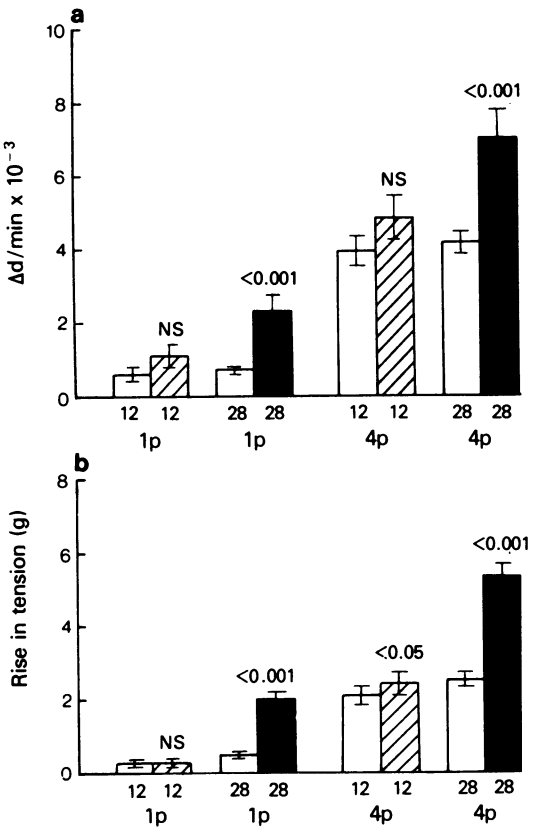

Figure 1 Effects of phenoxybenzamine on efflux of tritium and on mechanical responses in guinea pig vas deferens. (a) Stimulation-induced efflux in first stimulation period (open columns) in two sets of vasa at 1 and 4 pulses (1p and 4p respectively) and second stimulation periods in the absence (hatched columns) and presence (filled columns) of phenoxybenzamine (3.3×10^{-5} M). (b) The simultaneously recorded mechanical responses of the same preparations shown directly above in (a). Number of stimulation pulses and number of values are shown below columns. Data were analyzed by *t* test for paired data and probability comparisons (*P* values shown above second columns) are between first and second stimulation-induced effects in each set of vasa.

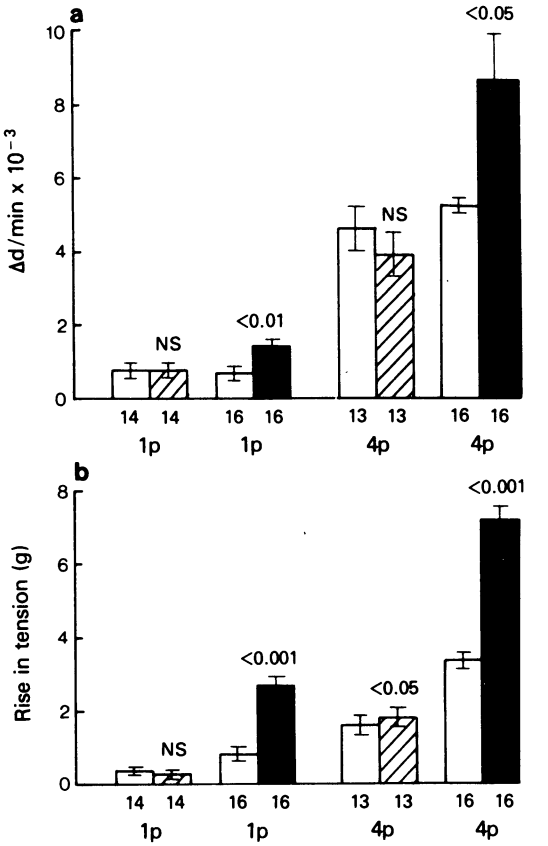

Figure 2. Effects of phenoxybenzamine on efflux of tritium and on responses in vas deferens after blockade of neuronal and extraneuronal uptake. All preparations were pretreated with cocaine (8.8×10^{-6} M) and hydrocortisone (2.8×10^{-5} M). (a) Stimulation-induced efflux in first stimulation period (open columns) in two sets of vasa at 1 and 4 pulses (1p and 4p respectively) and second stimulation periods in the absence (hatched columns) and presence (filled columns) of phenoxybenzamine (3.3×10^{-5} M). (b) The simultaneously recorded mechanical responses of the same preparations shown directly above in (a). Number of stimulation pulses and number of values are shown below columns. Data were analyzed by *t* test for paired data and probability comparisons (*P* values shown above second columns) are between first and second stimulation-induced effects in each set of vasa.

onist. The mechanical response was increased 413%. The outflow of labelled material and the mechanical response were also much increased above initial control values with four pulses (5 Hz), as anticipated.

Effect of neuronal and extraneuronal uptake blockade

The protocol described above was repeated in other preparations in which both neuronal and extraneuronal uptake processes were inhibited to eliminate the possibility that the observed effects of phenoxybenzamine are, in some way, linked to a diversion of [^{3}H]-noradrenaline from tissue inactivation sites. The output of tritium in response to one pulse, in the presence of hydrocortisone (2.8×10^{-5} M) and cocaine (8.8×10^{-6} M) was $0.75 \times 10^3 \pm 0.13$ d/min (30 values), which was not significantly different from that obtained in the absence of the inhibitors, a mean

of $0.69 \times 10^3 \pm 0.1$ d/min (40 values). Similarly, the mechanical responses to a single pulse in the absence and presence of the two inhibitors did not differ significantly; they were means of 0.4 ± 0.1 g and 0.6 ± 0.1 g in 40 untreated and 30 treated strips, respectively. Since blockade of tissue uptake sites did not enhance outflow or response, such an action by phenoxybenzamine cannot explain its effectiveness in promoting efflux of tritium under conditions of single pulse excitation. This was confirmed by direct analysis of the effects of phenoxybenzamine as shown in Figure 2.

The adrenoceptor antagonist significantly enhanced both the output of labelled noradrenaline and the mechanical response after single pulse excitation in vasa deferentia even in the presence of cocaine and the steroid. Not surprisingly, stimulation with four pulses (5 Hz) also yielded a substantially higher output of radioactivity after phenoxybenzamine treatment. Although the preparations not exposed to phenoxybenzamine showed a modest but significant increase in the mechanical response to four pulses on the second run compared to the first, those treated with the antagonist demonstrated a much larger increase; it was 214% compared to 113% for the control tissues.

Spontaneous efflux of tritium

The basal efflux of radioactivity was not significantly altered in tissues exposed to hydrocortisone (2.8×10^{-5} M) and cocaine (8.8×10^{-6} M). It was $12.8 \times 10^3 \pm 0.4$ and $11.7 \times 10^3 \pm 0.4$ d/min in 80 untreated and 60 treated strips, respectively. Phenoxybenzamine significantly increased the basal efflux of tritium both in untreated vasa deferentia and in those pretreated with the inhibitors of neuronal and extraneuronal uptake (Figure 3). In contrast, preparations not treated with phenoxybenzamine showed a significant decrease in spontaneous efflux between first and second runs in the presence of the two uptake antagonists.

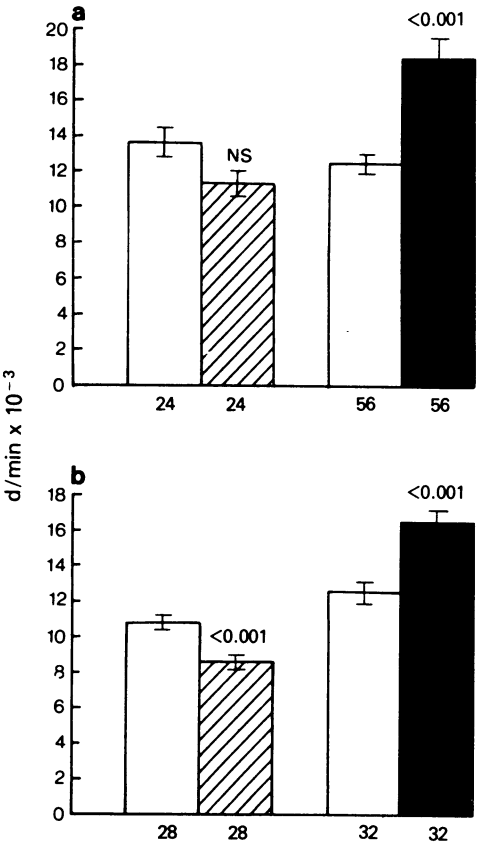

Figure 3 Basal efflux of tritium in untreated and treated vas deferens pre-incubated with [^{3}H]-noradrenaline. (a) Basal efflux in two sets of vasa prior to first run of nerve-induced stimulations (open columns) and before second run in the absence (hatched columns) and presence (filled columns) of phenoxybenzamine (3.3×10^{-5} M). (b) Same protocol as in (a), except all preparations were exposed to cocaine (8.8×10^{-6} M) and hydrocortisone (2.8×10^{-5} M) throughout the experiment. *P* values are shown above second column.

Discussion

Previous workers have shown that phenoxybenzamine enhances the stimulation-induced outflow of tritium in autonomic effectors pre-incubated with [^{3}H]-noradrenaline (see reviews by Rand, McCulloch & Story, 1975; Starke & Endo, 1976; Langer, 1977; Starke, 1977). The reproducibility of this observation in virtually all sympathetically-innervated systems examined, including the vas deferens, has been instrumental in the formulation of the hypothesis of presynaptic modulation of transmitter release (Haggendal, 1970; Langer, 1970; Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Starke, Montel & Schümann, 1971; McCulloch, Rand & Story, 1972). This hypothesis proposes that activation of presynaptic inhibitory receptors by noradrenaline released by nerve impulses or added exogenously, inhibits subsequent nerve-induced release and that phenoxybenzamine enhances transmitter outflow by blockade of these inhibitory receptors. A function served by this negative feedback system is presumed to be modulation of the magnitude of postsynaptic responses.

The present experiments were designed to determine if phenoxybenzamine promoted the efflux of neurotransmitter under experimental conditions

which preclude participation of a presynaptic negative feedback system; that is single pulse stimulation of the effector. The concentration of phenoxybenzamine selected for use (33 μM) is in the range used by others (7 to 66 μM) to achieve a maximal or near maximal increase in transmitter efflux (Kirpekar & Puig, 1971; Farnebo & Hamberger, 1971; Farnebo & Malmfors, 1971; Stjärne, 1973; Hughes & Roth, 1974; Rand *et al.*, 1975; Langer, 1977) and response magnitude (Burn & Gibbons, 1964). This was deemed important to ensure that the presence or absence of an increase in tritium efflux and response magnitude with one pulse, where the absolute stimulation-induced efflux is small, could be clearly determined. Phenoxybenzamine at 30 μM has been shown to enhance stimulation-induced release in guinea-pig vas deferens and this is coupled with an extrusion of increased amounts of soluble dopamine β-hydroxylase, supporting the concept of an enhanced exocytotic extrusion of transmitter by the antagonist at the concentration used here (Johnson, Thoa, Weinshilboum, Axelrod & Kopin, 1971).

It was found that the adrenoceptor antagonist significantly enhanced the overflow of tritium when only a single pulse of 1 ms duration was administered by field stimulation to the vas deferens preparation and when neuronal and extraneuronal pathways of agonist diversion were excluded. Thus, it appears that the action of phenoxybenzamine, at least in the vas, cannot be simply encompassed within a unitary hypothesis that proposes modulation of sympathetic neurotransmitter release by previously released noradrenaline.

The possibility that noradrenaline released initially during a single pulse may inhibit further release by that same pulse probably can be dismissed. Katz & Miledi (1968) using the frog sartorious nerve-muscle preparation found that the inward movement of calcium occurs during depolarization, as does the rise in intracellular 'active' calcium, and that these events are essentially terminated with the end of the action potential. These workers have pointed out (1967) that the release of transmitter (in their case acetylcholine) lags behind the depolarization which causes it, starting after the end of a pulse of less than 4 ms duration.

Two phases of calcium entry into the neurone occur during the action potential. The late phase, which appears to be particularly relevant to adrenergic transmitter release (Aguirre, Pinto & Trifaró, 1977), turns on at the same time as the increase in potassium permeability (Baker, 1972). As pointed out by Baker, depolarization has a dual action serving both to activate and inactivate calcium entry.

Rahamimoff (1970) notes that 'Applying calcium immediately after the depolarizing pulse does not produce any release of transmitter. Such experiments show that calcium action is very rapid on a biological time scale, and that it must be present either before or during the depolarization. It is ineffective if applied after the depolarization, even before the onset of transmitter liberation, that is, during the synaptic delay'. It appears then that membrane events as well as the intraneuronal increase in 'active' calcium, relevant to release, are over at the time of exocytotic expulsion of transmitter.

Sensitization of mechanical responses to sympathetic nerve stimulation by phenoxybenzamine and other adrenoceptor antagonists has been observed in a number of preparations with predominantly β-receptors and also in certain tissues with α-receptor mediated responses, such as the vas deferens, pulmonary artery, and spleen (Kirpekar & Cervoni, 1963; Burn & Gibbons, 1964; Langer, Adler-Graschinsky & Giorgi, 1977; Blakeley & Summers, 1978). This is interpreted by proponents of the presynaptic receptor hypothesis to represent the anticipated consequences of blockade by the antagonists of the presynaptic negative feedback system (e.g. Rand *et al.*, 1975; Langer, 1977), in tissues where postsynaptic receptor blockade does not obliterate its observation. However, the present finding that the mechanical response to single pulse excitation was enhanced, although continuing to support a possible association between phenoxybenzamine-induced efflux and the magnitude of the effector contraction, cannot be explained on the basis of a presynaptic receptor system engaged by endogenously released noradrenaline.

Spontaneously released transmitter is composed of about 90% metabolites of [^{3}H]-noradrenaline, almost all of which are believed to represent transmitter deaminated intraneuronally prior to release (Langer & Enero, 1974; Cubeddu, Barnes, Langer & Weiner, 1974; Brandao, 1977). A portion of the remaining tritium probably derives from extraneuronal sources, at least in rabbit aorta (Eckert, Henseling & Trendelenburg, 1976). The basal efflux of tritium was increased by phenoxybenzamine in the vas deferens, pointing to an action of the haloalkylamine on some aspect of neuronal release. Whether this is a non-specific intraneuronal effect of the antagonist or related to a specific receptor is not clear, but this same action also may be involved in the stimulation-enhanced efflux of tritium. In this connection, it should be noted that Rand, Story, Allen, Glover & McCulloch (1973) found that phenoxybenzamine does not increase the efflux of tritium after a single pulse administered to guinea-pig atria. Whether the differences between their observations and the present ones reflect organ specific differences or details of experimental design is not clear.

It was expected, in accordance with presynaptic receptor theory, that the efflux of tritium following four pulses (5 Hz) would be enhanced by phenoxybenzamine, since it could be reasonably postulated

that at least some minimal accumulation of noradrenaline in the 'biophase' occurs between pulses. Both enhanced efflux and magnified mechanical responses were observed. However, the relative outputs of tritium at one and four pulses failed to support the operation of an auto-inhibitory feedback system in the guinea-pig vas deferens. Rand *et al.* (1973; 1975) had proposed that, in systems which utilize a negative feedback system, a decrease in efflux per pulse with an increasing number of pulses, that is 'pulse-to-pulse' modulation of transmitter release, should be evident. However, in the present experiments the output at four pulses, in the presence of inhibitors of neuronal and extraneuronal uptake, was 4.9 times that at 1 pulse in the same tissues and this ratio was not significantly modified by phenoxybenzamine.

The mediator of contractile responses to field stimulation in the vas deferens of guinea-pig and other species is controversial, with some workers suggesting that noradrenaline, alone, cannot account for responses (Ambache & Zar, 1971; von Euler & Hedqvist, 1975; Westfall, Stitzel & Rowe, 1978). However, this tissue has been basic to the development of the concept of presynaptic receptors, since findings made with it generally extend to other preparations (see reviews by Starke, 1977; Langer, 1977) and it continues to be widely used to enrich the theory and its implications (Rand *et al.*, 1975; Stjärne, 1975; Langer, 1977; Marshall, Nasmyth & Shepperson, 1978; Drew, 1978; Harper & Hughes, 1978).

The observations recorded here require that the presynaptic receptor hypothesis, in its present form, be subjected to a more rigorous scrutiny than it has been up to now and that other explanations for the observed effects of agonists and antagonists on [^{3}H]-transmitter release be considered.

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