

Direct evidence against a role of ATP as the nonadrenergic, noncholinergic inhibitory neurotransmitter in guinea pig tenia coli

(P₂ purinergic receptors/arylazido aminopropionyl ATP/photoaffinity label/nonadrenergic, noncholinergic nerves/purinergic nerves)

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ABSTRACT Electrical field stimulation of the isolated guinea pig tenia coli in the presence of a muscarinic receptor antagonist (atropine) and an adrenergic neuron blocker (guanethidine) produces relaxation. A large amount of indirect evidence has suggested that the neurotransmitter that is released from these nonadrenergic, noncholinergic inhibitory neurons is ATP or a related nucleotide, and the nerves have been termed "purinergic." A photoaffinity analog of ATP, arylazido aminopropionyl ATP, which produces a specific pharmacological antagonism of P₂ purinergic receptors in isolated guinea pig vas deferens and urinary bladder, was utilized in the present study to evaluate directly whether ATP is the nonadrenergic, noncholinergic inhibitory neurotransmitter in tenia coli. By blocking postjunctional P₂ receptors, arylazido aminopropionyl ATP produced a pronounced antagonism of relaxations induced by exogenously added ATP. Responses produced by ADP, AMP, and adenosine also were antagonized by arylazido aminopropionyl ATP, but to a lesser extent. Inhibitory responses to isoproterenol were not antagonized. Under these conditions of established, specific P₂-receptor blockade of responses to exogenously added ATP, relaxations induced by field stimulation of intrinsic inhibitory nerves in the presence of atropine (1 μM) and guanethidine (1 μM) were not antagonized. Though these results provide no indication of the actual substance involved, they suggest strongly that the nonadrenergic, noncholinergic inhibitory neurotransmitter in the guinea pig tenia coli is not ATP.

Responses of many autonomically innervated smooth muscle preparations to stimulation of intrinsic nerves are not blocked by conventional agents that should antagonize the effects of neurotransmitters which are released from adrenergic or cholinergic nerves. Thus, relaxation of the smooth muscle of isolated tenia coli (1), trachealis (2–6), and anococcygeus (7) preparations results from electrical field stimulation even if an adrenergic neuron blocker such as guanethidine or adrenoceptor antagonists (or both) are present. Likewise, contractions of urinary bladder evoked with field stimulation contain a substantial cholinergic component (8–12) but nevertheless are quite resistant to the muscarinic antagonist atropine. It has been proposed mainly by Burnstock *et al.* (1, 13) that the "nonadrenergic, noncholinergic" motor nerves that innervate these tissues use ATP or a related nucleotide as their primary neurotransmitter. Conventional antagonists are ineffective against responses mediated by these "purinergic" nerves (1, 13, 14) because they are mediated *via* the interaction of ATP or its breakdown product, adenosine (15, 16), acting through subclasses of what were described by Burnstock (17) as "purinergic" receptors.

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The evidence in favor of the purinergic nerve hypothesis is compelling but indirect. It has been reviewed periodically (13, 14, 18). Many investigators (3, 19–27) have shown that agents—none of them specific receptor blockers—that modify responses to exogenously added ATP can alter in a similar manner neurogenically induced responses. Direct evidence demonstrating a functional, neurotransmitter role of ATP would be provided by evaluation of neurogenic responses obtained in preparations in which responses to exogenously added compound are antagonized by blockade of postjunctional purinergic receptors. An antagonist of this type has not been available until recently.

We recently have determined that in the presence of isolated tissues, photolysis of arylazido aminopropionyl ATP [arylazido NH₂Pp-ATP; 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl} adenosine 5'-triphosphate] (28)—a photoaffinity analog of ATP—produces a specific pharmacological antagonism of ATP-induced contractile responses of the vas deferens of guinea pigs (29), rats (30), and rabbits (unpublished) and of the urinary bladder of guinea pigs (31), rabbits, and humans (unpublished). Neurogenic responses also were antagonized by arylazido NH₂Pp-ATP, and a functional cotransmitter role of ATP or related nucleotides has been established for these organs (31, 32). These antagonisms result from the photolysis-dependent formation of a covalent bond at or near the P₂ receptor.

The purpose of the present study was 2-fold: (i) to determine if arylazido NH₂Pp-ATP can antagonize nucleotide-induced relaxation of the guinea pig tenia coli, which, like the aforementioned preparations, is mediated by P₂ receptors and (ii) to determine if blockade of P₂ receptors affects the relaxation of the tissue in response to stimulation of the nonadrenergic, noncholinergic nerves. A preliminary account of this work has appeared (33).

MATERIALS AND METHODS

Tenia coli were removed from male guinea pigs (English short-hair; Hilltop Lab Animals, Scottsdale, PA) that were killed by a blow to the head. Segments of tissue (10–15 mm long) were tied to a holder, placed in a glass, water-jacketed (37°C) 2-ml organ chamber, through which modified Krebs–Henseleit solution (32) was suffused (inlet, bottom; outlet, top), and attached to a transducer for the measurement of isometric tension responses. The Krebs–Henseleit solution always contained 1 μM atropine and 1 μM guanethidine. Resting tension was 0.5 g. The tissues were suffused continuously for 45 min before experiments were begun.

Abbreviation: arylazido NH₂Pp-ATP, arylazido aminopropionyl ATP (3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl} adenosine 5'-triphosphate); abbreviated as "ANAPP₃" in previous reports.

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Arylazido $\text{NH}_2\text{Pp-ATP}$ was synthesized according to Jeng and Guillory (28). Treatment of the tissues with arylazido $\text{NH}_2\text{Pp-ATP}$ was as follows. Arylazido $\text{NH}_2\text{Pp-ATP}$ (0.1 mM) was added to the organ bath containing the tenia coli and, after a 3-min incubation, the preparations were irradiated for 30 min with a high-intensity projector lamp (DVY; 650 W; 3,400 K), the filament of which was 7 cm from the center of the chamber. Bath temperature remained at 37°C during irradiation. After photolysis the arylazido $\text{NH}_2\text{Pp-ATP}$ was washed from the bath with fresh Krebs–Henseleit solution. Control preparations were irradiated in the absence of arylazido $\text{NH}_2\text{Pp-ATP}$ according to this procedure. The pharmacological experiments were begun 5 min later.

The relationship of tension responses to the concentration of exogenously added agonists (e.g., adenine compounds or isoproterenol) was determined in preparations that were allowed to maintain slow, rhythmic contractions or in others in which tone was induced by addition of $3\ \mu\text{M}$ histamine to the suffusing medium. In the former case, suffusion was stopped and an agonist was added to the bath at the peak of the spontaneous contraction. After 15 sec, the drug was removed by suffusion. When tone was induced with histamine, suffusion was stopped and the tissue was exposed to agonist for 15 sec, with 2.75-min suffusion periods between additions. Each preparation was used for only one stepwise-increasing, noncumulative concentration–response determination.

To study responses to electrical field stimulation, tissues were mounted inside two adjacent platinum ring electrodes placed 1 cm apart along the longitudinal axis of the muscle. Rectangular wave pulses (40 V, 0.5-msec duration) were delivered between the electrodes in 10-sec trains of varying frequency. The tissues were stimulated at the peak of spontaneously developed contractions during continuous suffusion or at 3-min intervals in preparations that were first made to contract with histamine ($3\ \mu\text{M}$) present in the suffusing medium. Only one frequency–response relationship was evaluated in each preparation. Responses to electrical field stimulation were abolished in the presence of tetrodotoxin ($1\ \mu\text{M}$; $n = 5$) and therefore were neurogenic in nature.

Drugs and adenine compounds were from Sigma. The results are presented as means \pm SEM. Differences were evaluated with Student's *t* test for nonpaired data and $P < 0.05$ was taken to indicate statistical significance. n is the number of separate experiments.

RESULTS

The relative potency series for adenine compounds that is characteristic of P_2 receptors (14) was observed in tissues made to contract with histamine (Fig. 1). In order of decreasing potency, the rank order was $\text{ATP} > \text{ADP} > \text{AMP} > \text{adenosine}$, and the respective ED_{50} values were $0.11\ \mu\text{M}$, $0.46\ \mu\text{M}$, $17.45\ \mu\text{M}$, and $42.0\ \mu\text{M}$. Each agent produced an equivalent maximal relaxation response insofar as the tissues were relaxed to baseline tension in each case. However, the duration of the relaxation was greatest for ATP and was the least for adenosine (not shown).

The effect of photolytic treatment of tissues with 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on responses of the tissues to exogenously added agonists is summarized in Fig. 2, in which it may be seen that concentration–response curves for ATP, ADP, AMP, and adenosine were shifted in a parallel manner to the right of control; maximal responses were unaffected. The shifts of the ED_{50} values for ATP, ADP, AMP, and adenosine were 6-, 2.4-, 3.8-, and 2.4-fold, respectively; they were significant in each case. These results indicated that responses to ATP were the most readily antagonized.

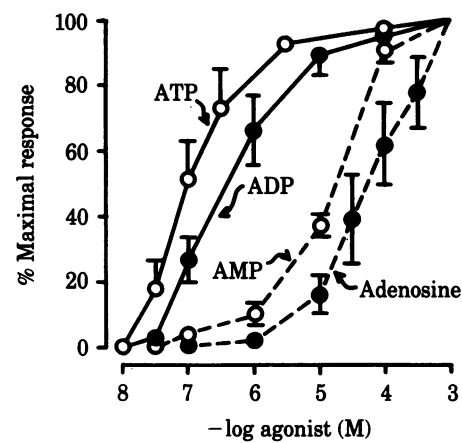


FIG. 1. Concentration–response curves for relaxation by adenine nucleotides and adenosine of tenia coli in the presence of $3\ \mu\text{M}$ histamine to induce tone. $n = 5-7$.

To determine whether the antagonism by arylazido $\text{NH}_2\text{Pp-ATP}$ resulted from blockade of specific receptors (29), its effect on responses to isoproterenol, a β -adrenoceptor agonist, was evaluated. The concentration–response curve for isoproterenol

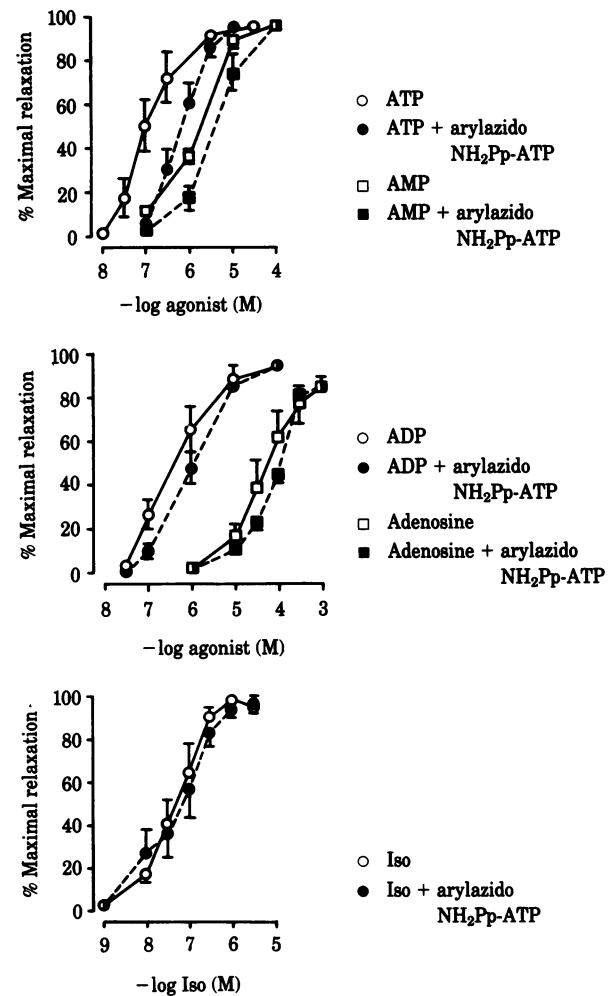


FIG. 2. Concentration–response curves for adenine nucleotides, adenosine, and isoproterenol (Iso) in irradiated control preparations (open symbols) and after photolytic treatment with 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ (closed symbols). $n = 6$ for ATP, 7 for ADP, 6 for AMP, 5 for adenosine, and 4 for Iso.

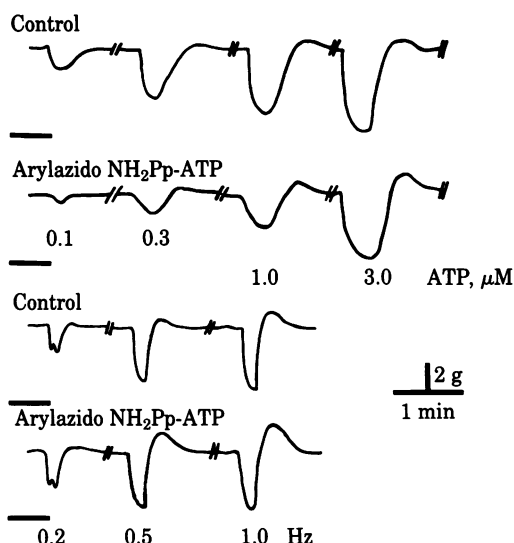


FIG. 3. The effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on responses to exogenously added ATP in preparations with tone induced with $3 \mu\text{M}$ histamine is shown in the upper two tracings. The effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on responses to electrical field stimulation in preparations with tone induced with $3 \mu\text{M}$ histamine is shown in the lower two tracings. The horizontal bars indicate 1 min.

was not significantly affected by arylazido $\text{NH}_2\text{Pp-ATP}$ treatment (Fig. 2). These findings indicate that arylazido $\text{NH}_2\text{Pp-ATP}$ is a specific and irreversible antagonist of adenine nucleotide-induced responses in a tissue in which they evoke relaxation—i. e., the antagonism by arylazido $\text{NH}_2\text{Pp-ATP}$ occurs at P_2 receptors whether they mediate contraction or relaxation.

The ability of arylazido $\text{NH}_2\text{Pp-ATP}$ to modify neurogenic responses was examined under conditions in which responses to exogenously added nucleotides were antagonized. The results of a representative experiment in which tone was induced with histamine are illustrated in Fig. 3. Responses of untreated tissues to exogenously added ATP and to field stimulation were similar in profile. Although the ability of arylazido $\text{NH}_2\text{Pp-ATP}$ to antagonize ATP-induced relaxation is evident, Fig. 3 shows that arylazido $\text{NH}_2\text{Pp-ATP}$ had no effect on responses to electrical field stimulation. The magnitude of the relaxations, the time courses, and the overall appearance of the responses were identical to those of the control preparations.

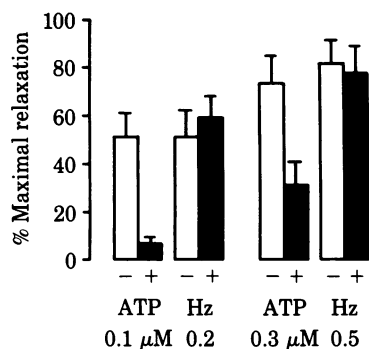


FIG. 4. Comparison of the effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on matched responses to exogenously added ATP and to electrical field stimulation in preparations with tone induced with $3 \mu\text{M}$ histamine. -, Irradiated controls; +, treated with arylazido $\text{NH}_2\text{Pp-ATP}$. Arylazido $\text{NH}_2\text{Pp-ATP}$ decreased ($P < 0.05$) responses to ATP but did not decrease ($P > 0.05$) those to field stimulation. $n = 6$.

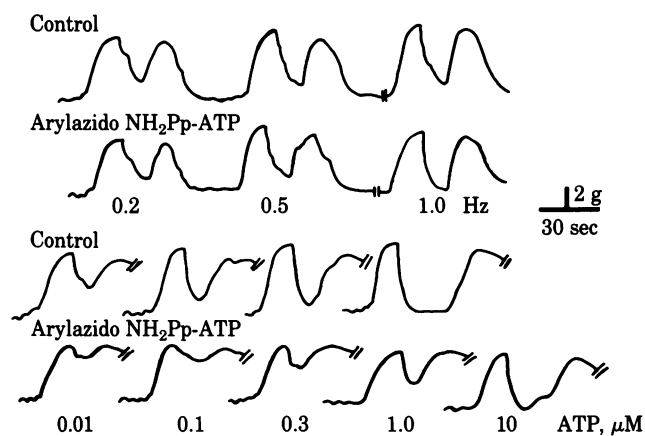


FIG. 5. The effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on responses to electrical field stimulation in preparations generating spontaneous contractions is shown in the upper two tracings. The effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on responses to ATP in preparations generating spontaneous contractions is shown in the lower two tracings. Calibration time is different than that of Fig. 3.

Fig. 4 shows a direct comparison between responses to ATP and to field stimulation produced by concentrations and stimulation frequencies that evoke similar degrees of relaxation. It is clear from this summary that although responses to exogenously added ATP were decreased markedly by arylazido $\text{NH}_2\text{Pp-ATP}$, the responses to field stimulation were unaffected.

It seemed possible that the induction of a constant level of tone with histamine, though easing the experimental procedures, could have interfered with neurotransmission or masked a possible effect of arylazido $\text{NH}_2\text{Pp-ATP}$ in some other way. To validate this lack of effect of arylazido $\text{NH}_2\text{Pp-ATP}$ on neurogenic responses, we repeated these experiments in the absence of histamine and we stimulated the tissues electrically or with ATP at the peak of the spontaneously generated contractions. Representative results are given in Fig. 5. Under these conditions, ATP and electrical stimulation produced concentration- and frequency-related relaxation responses. As was the case when tone was induced with histamine, arylazido $\text{NH}_2\text{Pp-ATP}$ treatment antagonized responses to ATP. Consistent with the earlier findings, relaxations caused by field stimulation were not modified in preparations treated with arylazido $\text{NH}_2\text{Pp-ATP}$. These results are summarized in Fig. 6, in which matched responses to ATP and to field stimulation are compared to emphasize that the inability of arylazido $\text{NH}_2\text{Pp-ATP}$ to antagonize

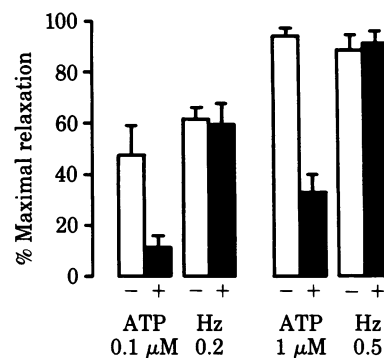


FIG. 6. Comparison of the effect of photolyzed 0.1 mM arylazido $\text{NH}_2\text{Pp-ATP}$ on matched responses to exogenously added ATP and to electrical field stimulation in preparations generating spontaneous contractions. - and + as in Fig. 4. Arylazido $\text{NH}_2\text{Pp-ATP}$ decreased ($P < 0.05$) responses to ATP but did not decrease ($P > 0.05$) those to field stimulation. $n = 5$.

relaxations that were induced neurogenically was unrelated to the magnitude of response.

DISCUSSION

One broad conclusion to be reached from this study is that photolyzed arylazido $\text{NH}_2\text{Pp-ATP}$ is a specific and irreversible P_2 -receptor antagonist in a system in which stimulation of the receptor triggers relaxation. Arylazido $\text{NH}_2\text{Pp-ATP}$ antagonized responses to ATP, ADP, and AMP but did not antagonize those to isoproterenol.

It was of concern that arylazido $\text{NH}_2\text{Pp-ATP}$ also antagonized to some extent responses to adenosine, because this suggests that arylazido $\text{NH}_2\text{Pp-ATP}$ might have interacted with and blocked P_1 receptors that could have mediated, in part, relaxation caused by the nucleoside. A crossover in receptor specificity would place a limitation on the general applicability of arylazido $\text{NH}_2\text{Pp-ATP}$. This possibility could not be assessed previously in tissues (see above) in which adenine nucleotides cause contraction for the reason that adenosine does not cause responses.

Results of other studies strongly support the view that responses of the tenia coli to adenosine that were antagonized by arylazido $\text{NH}_2\text{Pp-ATP}$ reflect an interaction of adenosine with the P_2 receptor rather than a blockade of the P_1 receptor by arylazido $\text{NH}_2\text{Pp-ATP}$. For example, Brown and Burnstock (34) provided evidence for the existence of both P_1 and P_2 receptors in the guinea pig tenia coli (also see ref. 35), which act as separate receptors for adenosine and ATP, respectively. They observed that theophylline, a P_1 -receptor antagonist, antagonized responses to adenosine but did not antagonize those to ATP. Sneddon *et al.* (7) have found that relaxations of the rabbit anococcygeus muscle produced by ATP and by adenosine were not blocked by arylazido $\text{NH}_2\text{Pp-ATP}$. ATP and adenosine were nearly equipotent and, on the basis of other experiments, it was determined that arylazido $\text{NH}_2\text{Pp-ATP}$ was ineffective against ATP because responses to the nucleotide followed its conversion to adenosine, which actually mediated the response. Similarly, Frew and Lundy (36) reported that arylazido $\text{NH}_2\text{Pp-ATP}$ was unable to antagonize ATP-induced relaxation of the guinea pig stomach fundus. Later studies indicated that these responses are mediated by P_1 receptors, because they are antagonized by 8-phenyltheophylline (ref. 37; R. Frew, personal communication); this implies that a conversion of ATP into adenosine occurs. Therefore, the cumulative evidence obtained by us and others thus far indicates that the antagonism by arylazido $\text{NH}_2\text{Pp-ATP}$ is highly specific for the P_2 receptor.

The second purpose of this study was to test the hypothesis that the transmitter that mediates responses resulting from stimulation of the nonadrenergic, noncholinergic motor nerves in the tenia coli is ATP or a related adenine nucleotide or adenosine. The evidence obtained does not support this hypothesis.

Arylazido $\text{NH}_2\text{Pp-ATP}$ readily blocked relaxations induced by exogenously added ATP in preparations with tone that was induced with histamine, or in others in which contractions developed spontaneously. Responses to field stimulation in the presence of both atropine—to block the excitatory action of acetylcholine released from cholinergic nerves—and guanethidine—to prevent the release of norepinephrine, which is inhibitory, from adrenergic nerves—were, under identical experimental conditions, totally insensitive to arylazido $\text{NH}_2\text{Pp-ATP}$.

It is possible that the light that penetrated the tenia coli was insufficient to photoactivate arylazido $\text{NH}_2\text{Pp-ATP}$ in the bio-phase of the inner layers of smooth muscle. There is little likelihood that this consideration explains the observations because photolyzed arylazido $\text{NH}_2\text{Pp-ATP}$ effectively antagonizes neu-

rogenic contractions of guinea pig vas deferens (32) and urinary bladder (31), and cotransmitter roles for ATP in these tissues have been suggested. The motor nerves innervating these organs penetrate all layers of muscle, and the bladder wall is considerably thicker than the tenia coli. Because the pharmacological antagonism of P_2 receptors does not modify the neurogenic response in the presence of atropine and guanethidine, this study provides direct evidence against the hypothesis that suggests a transmitter role for ATP in the guinea pig tenia coli.

This study also suggests that adenosine formed from the rapid enzymatic breakdown of neurally released ATP probably is not involved as a transmitter. In systems in which this process has been documented [e.g., guinea pig trachealis (25) and stomach fundus and rabbit anococcygeus (7)], responses to ATP are antagonized by methylxanthines, and ATP and adenosine have similar potency. Moreover, the antagonism of responses to exogenously added ADP, AMP, and adenosine by arylazido $\text{NH}_2\text{Pp-ATP}$ (Fig. 2) was of sufficient magnitude to have allowed resolution of the possible postjunctional role of these compounds in neurogenic responses, were they to have been formed. As such, our findings leave open the question of the identity of the nonadrenergic, noncholinergic mediator. The relationship between our findings and the strong evidence in favor of the purinergic nerve hypothesis is not readily apparent.

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