Br. J. Pharmac. (1972), **44**, 451–461.

Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide

G. BURNSTOCK, B. DUMSDAY AND A. SMYTHE

Department of Zoology, University of Melbourne, Parkville, 3052, Victoria, Australia

Summary

1. The possibility that a purine nucleotide is involved in excitatory transmission to the urinary bladder has been tested. All the purine compounds tested which contained a pyrophosphate bond produced contraction, adenosine triphosphate (ATP) being the most potent. Adenosine and adenosine monophosphate caused relaxation.

2. The response to ATP closely mimicked the nerve-mediated contraction, both being characterized by a rapid contraction which was not maintained. A lack of sensitivity to ATP was noted in some preparations of the rat urinary bladder.

3. Both nerve-mediated contractions and contractions caused by ATP were blocked by quinidine, while the response to acetylcholine persisted.

4. Nerve-mediated responses were depressed during tachyphylaxis produced by high concentrations of ATP. Tachyphylaxis did not occur when low concentrations were used. Possible explanations for these results are discussed.

5. The results are consistent with the hypothesis that non-cholinergic excitatory nerves to the guinea-pig bladder release a purine nucleotide, but do not provide critical evidence for it.

Introduction

The urinary bladder of all vertebrates studied receives an excitatory innervation via the pelvic nerves (see Burnstock, 1969). The nature of the transmitter substance released from excitatory nerves supplying the bladder has not been clearly demonstrated. In all species studied the responses to nerve stimulation are not abolished by muscarinic receptor blockade (for example in toad, Burnstock, O'Shea & Wood, 1963 ; lizard, Burnstock & Wood, 1967 ; marsupial, Burnstock & Campbell, 1963 ; and in placental mammals, Gruber, 1933). The nerve-mediated responses are, however, enhanced by anticholinesterase drugs (Edge, 1955) and are slightly reduced by atropine even in low doses. Increasing the dose of atropine does not decrease the response further (Ursillo, 1961). Thus a part of the bladder innervation may be cholinergic. Despite the failure of atropine to produce blockade, it has frequently been proposed that the nerves are entirely cholinergic. Arguments have been put forward to indicate under what conditions the nerves could be cholinergic yet atropine resistant (for example, Hukovic, Rand & Vanov, 1965 ; but see Dumsday, 1971).

The alternative explanation, accepted by several authors, is that the atropine resistant responses are due to nerves that are not cholinergic (Henderson & Roepke, 1934; Ambache & Zar, 1970; Dumsday, 1971). This paper tests the suggestion that adenosine triphosphate or a related nucleotide is the transmitter substance producing the atropine resistant contraction of urinary bladder (Dumsday, 1971). It has already been shown that adenosine triphosphate causes a contraction of dog and cat urinary bladder (Buchthal & Kahlson, 1944; Matsumura, Taira & Hashimoto, 1968). Furthermore it has been shown that other autonomic nerves release a purine nucleotide, probably adenosine triphosphate, as a transmitter substance (Burnstock, Campbell, Satchell & Smythe, 1970).

Methods

The urinary bladders of rats and guinea-pigs were used because it is evident that adrenergic innervation is weak, if not absent. The bladder preparations were isolated, bisected in the sagittal plane and one-half suspended in McEwen's (1956) or Krebs solution at 35° C. Records were made on a smoked kymograph drum via an isotonic frontal-writing lever. The nerves in the bladder wall were stimulated at 1–10 Hz with supramaximal pulses of 0·2–1 ms duration for periods of 10 s via platinum ring electrodes placed around the preparations. Drugs used were acetylcholine chloride (ACh), adenosine, adenosine-5′-monophosphate disodium salt (AMP), adenosine-5′-diphosphate monosodium salt (ADP), adenosine-5′-triphosphate disodium salt (ATP), ethylene-diamine-tetra-acetic acid disodium salt (EDTA), guanosine, guanosine-5′-monophosphate disodium salt (GMP), guanosine-5′-triphosphate trilithium salt (GTP), histamine acid phosphate, inosine, inosine-5′-monophosphate disodium salt (IMP), inosine-5′-triphosphate trisodium salt (ITP), quinidine hydrochloride, tetrodotoxin.

Results

Effects of electrical stimulation

Electrical stimulation of the rat or guinea-pig urinary bladder resulted in a strong, rapid contraction, beginning within 0·5 s of the onset of stimulation. At all stimulus frequencies used, the contraction was not maintained, beginning to decline from the maximum amplitude reached after 5–10 s of stimulation. A single pulse elicited a twitch-like contraction having one-fifth of the amplitude of a maximal tetanic response. The tetanic contraction produced by a train of pulses and the twitch-like contraction caused by a single pulse were each abolished by tetrodotoxin (5×10^{-7} g/ml). Responses to electrical stimulation are evidently entirely due to nerve mediated excitation, there being no evidence of direct muscle stimulation.

Effects of purines

Addition of adenosine triphosphate to the bathing fluid caused a contraction of the bladders of both species which began less than 0·5 s after adding the drug to the bath. The contraction was not maintained, but started to fade 3–5 s after the drug was applied. Tetrodotoxin (5×10^{-7} g/ml) had no effect on the contractions produced by adenosine triphosphate, indicating that adenosine triphosphate does not act by initiating nerve impulses. The threshold dose for a response of the guinea-pig bladder to adenosine triphosphate was 1–5 μM (0·6–3 μg/ml). In contrast, the

minimum dose of adenosine triphosphate required to produce a contraction of the rat bladder varied greatly, being 6 μM (10 μg/ml) in a few preparations, but in other rat bladders doses of adenosine triphosphate up to 330 μM (200 μg/ml) failed to produce a response. The response to adenosine triphosphate mimicked that to tetanic nerve stimulation. Both stimuli produced a contraction after a latent period of less than 0·5 s and the contractions faded rapidly after 3–5 seconds. In contrast, addition of acetylcholine to the organ bath produced a contraction which had a notably long latency of about 1 s ; the contraction faded very slowly until the drug was washed out of the organ bath.

The effects of a series of compounds related to adenosine triphosphate were tested on the guinea-pig bladder only. Adenosine diphosphate (1 mM ; 575 μg/ml) produced a contraction 30–50% of the amplitude of the contraction produced by 1 mM adenosine triphosphate ; the threshold dose for adenosine diphosphate was 20 μM. In contrast, 1 mM adenosine monophosphate or adenosine produced a slowly developing relaxation of the bladder (Fig. 1) ; neither adenosine monophosphate nor adenosine caused a contraction of the bladder at any concentration tested. Both guanosine triphosphate and inosine triphosphate produced contractions of the guinea-pig bladder at concentrations of 1 mM. The contractions rose to 50–60% of the amplitude of the response to an equimolar concentration of adenosine triphosphate (Fig. 1). No response was obtained with doses up to 1 mM of inosine monophosphate, guanosine monophosphate, inosine or guanosine.

Effects of inorganic phosphates and a chelating agent

Addition of sodium dihydrogen orthophosphate (NaH_2PO_4) or of sodium pyrophosphate ($NaH_3P_2O_7$) produced no response of the guinea-pig bladder in concentra-

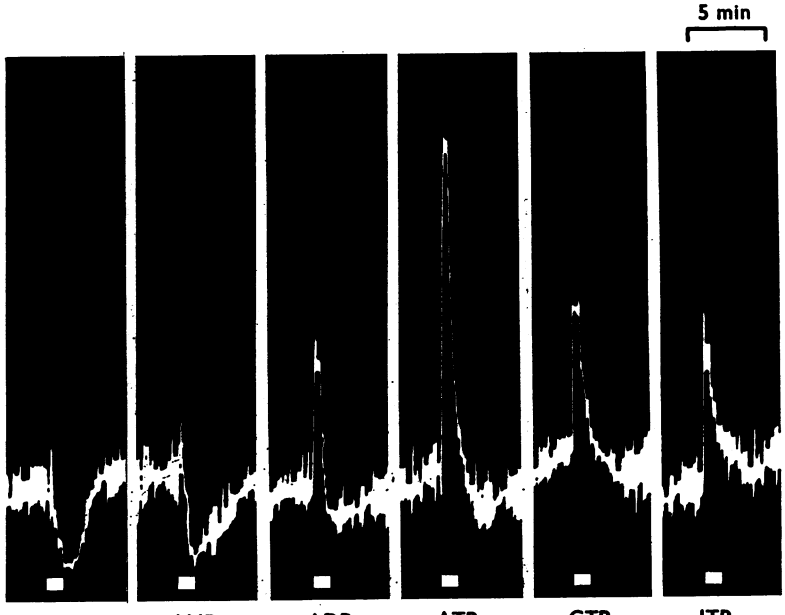

FIG. 1. Effects of purine compounds on isolated guinea-pig bladder. Bath concentration of each drug was 1 mM. The drugs were left in the bath until washed out after 23 seconds. In the first two panels note the long latency (20 s) before onset of the inhibitory responses to adenosine (ADEN) and AMP. In the remaining panels the contractions produced by addition of ADP, ATP, GTP and ITP, respectively, are shown. Time marker, 5 minutes.

tions up to 300 μM. The responses of the bladder produced by the active purines are clearly not due to the hydrolysis products of the nucleotides.

The chelating agent EDTA (200 μM) had no effect on the bladder, indicating that the responses produced by the nucleotides were not due to a chelating action of the purine nucleotides.

Effects of quinidine

The drug quinidine antagonizes the action of adenosine triphosphate on mammalian intestinal smooth muscle (Bowman & Hall, 1970; Burnstock *et al.*, 1970). Quinidine (10^{-4} g/ml) blocked the excitatory response of the rat and guinea-pig urinary bladder to concentrations of adenosine triphosphate up to 3×10^{-4} g/ml. In fact adenosine triphosphate in the same concentrations as those previously producing contraction, now caused a relaxation of the urinary bladders. Reversal of the response to adenosine triphosphate was achieved after exposure for 0·5–1 h to quinidine. Concentrations of quinidine causing reversal of the response to adenosine triphosphate also abolished the nerve mediated contractions of the bladder after 2 h (Fig. 2). Increasing the stimulating voltage did not restore the response. It was not possible, however, to demonstrate a relaxation of the bladder produced by nerve

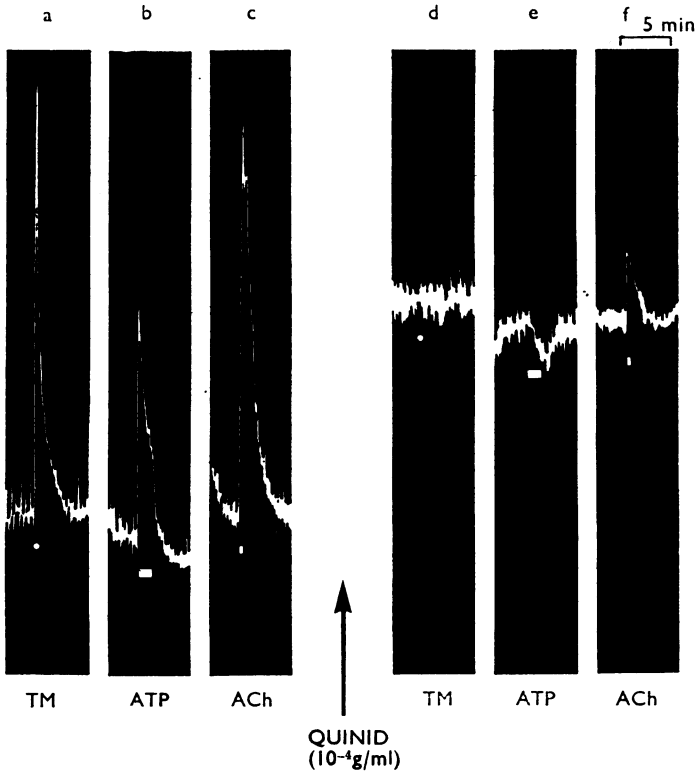

FIG. 2. Effects of quinidine on excitatory responses of isolated guinea-pig bladder to stimulation of the intramural nerves and to application of ATP and ACh. Panels a,b and c show responses to transmural stimulation at 5 Hz for 10 s (TM), ATP (3×10^{-4} g/ml) applied 90 s and ACh (3×10^{-5} g/ml) applied for 30 s respectively. Panels d, e and f show responses to the same stimuli obtained 3 h after the addition of quinidine (QUINID, 10^{-4} g/ml). Note that the response to intramural nerve stimulation is abolished, the contraction produced by ATP is blocked and in fact reversed to an inhibition, while the response to ACh is present though reduced. Time marker, 5 minutes.

stimulation after treatment with quinidine. The response to acetylcholine ($3{\cdot}2 \times 10^{-5}$ g/ml) was present after nerve blockade with quinidine, although reduced to 15% of the amplitude of the response obtained before treatment with quinidine. The responses obtained to stimulation by adenosine triphosphate, nerve excitation or acetylcholine before application of quinidine were fully restored by washing the

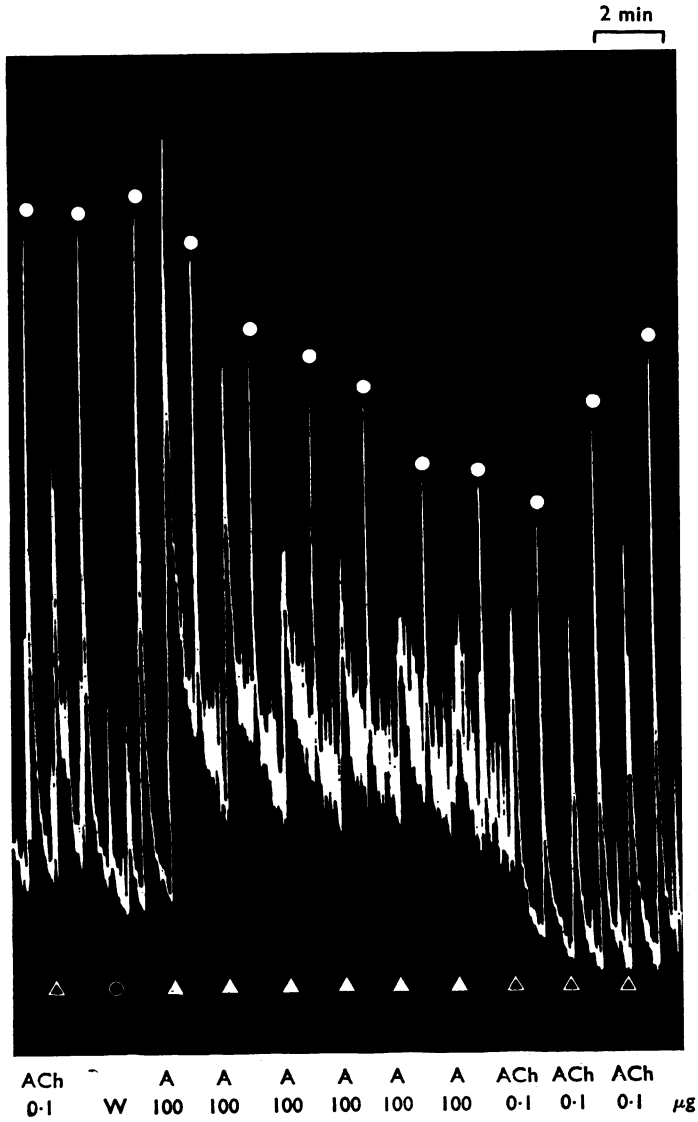

FIG. 3. Effects of accumulation of successive doses of ATP on the contractions of guinea-pig bladder induced by nerve stimulation, ACh and ATP. Transmural electrical pulses of 3 Hz for 5 s were applied at 2 min intervals throughout (white dots). ACh (10^{-7} g/ml at open triangles) was applied for 20 s, then washed out. The open circle indicates that washout only was performed. At the closed triangles ATP (10^{-4} g/ml, A) was added to the organ bath and not washed out. Note that responses to successive doses of ATP decline and that responses to nerve stimulations simultaneously decline. The response to the first subsequent dose of ACh, in the presence of ATP, is depressed. The responses to ACh and nerve stimulation after removal of the accumulated ATP increased and eventually (not shown) reached their respective control amplitudes. Numbers at the bottom of the figure indicate the dose added to the bath in μg/ml. Time marker, 2 minutes.

preparation in quinidine-free McEwen's solution for 2 hours. Quinidine (10^{-5} g/ml) had no effect on the responses to nerve stimulation or adenosine triphosphate. However, lower doses of quinidine (10^{-6} g/ml) enhanced responses to nerve stimulation, adenosine triphosphate and acetylcholine, and increased the amplitude of the spontaneous activity of the bladder.

Tachyphylaxis to adenosine triphosphate

The contractions of guinea-pig urinary bladder produced by adenosine triphosphate (6×10^{-5} g/ml) left in the organ bath for 10 s were of constant amplitude for successive doses of adenosine triphosphate applied at 5 min intervals. However, when the bath was not washed out between each dose of adenosine triphosphate (10^{-4} g/ml), a progressive decline of the amplitude of the contraction was seen (Fig. 3). When about six doses had accumulated, the contraction produced by adenosine triphosphate was at most only 20% of the initial response amplitude. At this time, contractions produced by nerve stimulation, acetylcholine (10^{-6} g/ml) or histamine (10^{-6} g/ml) were reduced to about 40% of the control amplitudes. Reduction of the responses to adenosine triphosphate and nerve stimulation by prolonged exposure to adenosine triphosphate is evidently due in part to a depressant effect on the muscle. These results apparently conflict with the observations of Ambache & Zar (1970), who have reported that tachyphylaxis to adenosine triphosphate may be established without reducing responses to nerve stimulation. Since Ambache & Zar used an experimental technique which differed from that used above, an attempt was made to duplicate their experiments, with the exception that the urinary bladder halves were left intact, whereas they stripped off the mucosa and serosa. In the present experiments, stripping of the mucosal and serosal layers did not produce an increase in the sensitivity of the bladder to agonist drugs.

In a first series of experiments nerve-mediated responses to single electrical pulses delivered at 2 min intervals were examined. Throughout the experiment the organ bath was washed out during the 10 s period beginning 90 s after an electrical pulse. After the control period a dose of acetylcholine (10^{-7} g/ml) was administered 60 s after an electrical pulse. Exposure to the drugs was terminated after 30 s by the washout. At 60 s after each successive pulse adenosine triphosphate was added to the organ bath. A dose of 10^{-6} g/ml of adenosine triphosphate in the organ bath was repeated until the presence or absence of any trend in the amplitudes of the nerve- and drug-elicited responses was established. The dose of adenosine triphosphate was then increased to give bath concentrations of 10^{-5} g/ml and finally 10^{-4} g/ml. The dose of acetylcholine used at the beginning was repeated at the conclusion of the experiment. In these experiments, tachyphylaxis to adenosine triphosphate if present was weak even when the dose of adenosine triphosphate was raised to 10^{-4} g/ml. The responses to nerve stimulation and to acetylcholine were not diminished during the experiment (Fig. 4). These results are at variance with those reported by Ambache & Zar.

A second series of experiments were performed, differing from the first series only in that the doses of adenosine triphosphate were not washed out of the organ bath. After four or five additions of the lowest dose of adenosine triphosphate used (10^{-6} g/ml), the responses to nerve stimulation and adenosine triphosphate were unchanged. When the dose was increased to 10^{-5} g/ml of adenosine triphosphate added to the organ bath, the response to each dose progressively declined. The

response to nerve stimulation decreased slightly. The dose of adenosine triphosphate added was again increased to give concentration increments of 10^{-4} g/ml in the bath. The response to the drug increased with the increase in dose then progressively declined for each succeeding dose. The response to nerve stimulation was also markedly depressed at this stage. However the response to acetylcholine was also depressed (Fig. 5). It can be seen that adenosine triphosphate tachyphylaxis was not produced in these experiments unless adenosine triphosphate was left in contact with the tissue; the results of Ambache & Zar were not verified.

Discussion

The experiments in this paper have been designed to test the suggestion that adenosine triphosphate, or a similar compound, might be a transmitter substance released by the excitatory innervation of the urinary bladder (Dumsday, 1971), as has been proposed for the non-adrenergic inhibitory nerves to the gut (Burnstock *et al.*, 1970). The results obtained are in many respects consistent with this hypothesis, but do not provide critical evidence for it. The supporting results are that the response to adenosine triphosphate mimics that to nerve stimulation, that quinidine blocks adenosine triphosphate and nerve mediated responses and that when tachyphylaxis to adenosine triphosphate is produced, nerve mediated responses are reduced.

The excitatory response to nerve stimulation has a rapid rising phase, and the preparation begins to relax after approximately 6 seconds. Thereafter, return to

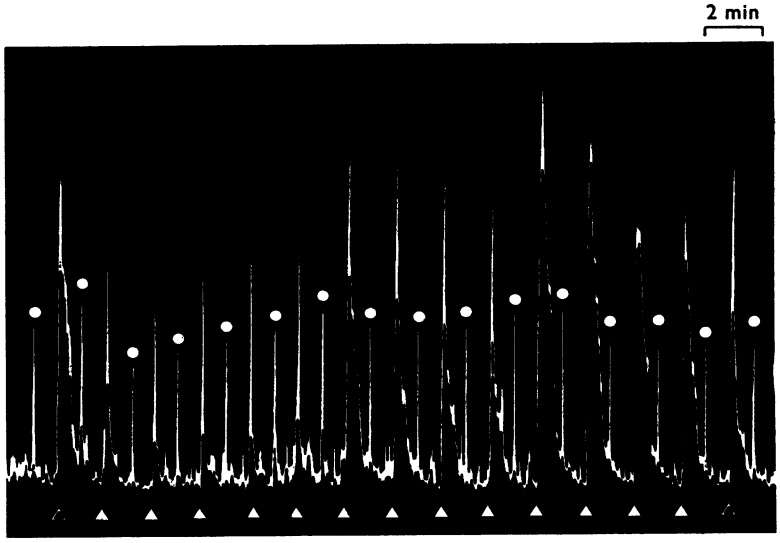

FIG. 4. Effects of repeated doses of ATP on the contractions of guinea-pig bladder produced by nerve stimulation and ATP. Single transmural electrical pulses (at white dots) delivered at 2 min intervals. Overflow washout was performed for 10 s beginning 90 s after each pulse. At the points marked by the open triangles, 60 s after a pulse, ACh (10^{-7} g/ml) was added to the organ bath. Exposure to the drug was terminated after 30 s by the washout. At 60 s after each successive pulse, ATP (A) was added to the organ bath at the points marked by closed triangles. The responses to repetitions of three different doses of ATP are shown. Note that no marked tachyphylaxis to ATP develops, although a slight reduction in amplitude of the responses to ATP does occur with the higher doses. The responses to nerve stimulation and ACh remain unchanged. Numbers at the bottom of the figure indicate the concentration in the organ bath of the added drug in μg/ml. Time marker, 2 minutes.

the level of tone seen before stimulation takes about 60 seconds. The time course of the nerve mediated response is similar for all frequencies of stimulation used. The latent period and time course of the excitatory response to exogenously applied adenosine triphosphate mimics the contraction produced by nerve stimulation. The similarity of the responses has been remarked upon by Ambache & Zar (1970). In contrast, acetylcholine is able to cause a prolonged excitation of the muscle after a longer latent period. The rapid decline of the nerve mediated response despite continued stimulation, indicates either that there is a declining release of transmitter during continued nerve excitation, or the transmitter which is released is not able to maintain an activation of the contractile mechanism. It can be seen that the decline of the response would be explained by assuming that adenosine triphosphate, which is not able to cause a prolonged response, is the transmitter substance. Although this explanation is not exclusive it does lend support to the view that adenosine triphosphate rather than acetylcholine is the transmitter substance. It should be noted that the inhibitory action of non-adrenergic inhibitory nerves and applied adenosine triphosphate on gut preparations are comparably short lasting (Burnstock *et al.*, 1970).

Quinidine prevents the inhibitory action of adenosine triphosphate on the rabbit ileum (Bowman & Hall, 1970) and the taenia of the guinea-pig caecum (Burnstock *et al.*, 1970). In the present experiments quinidine had a blocking action against the excitatory effects of adenosine triphosphate, and in fact reversed the response to

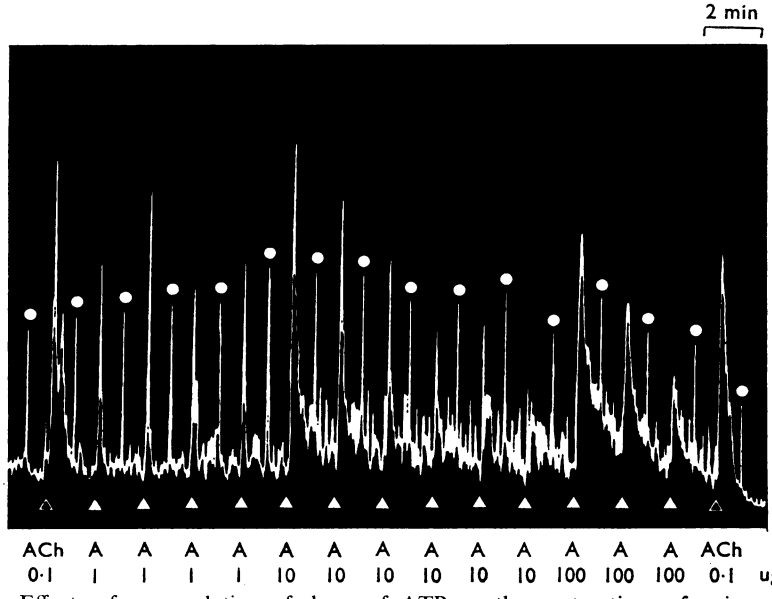

FIG. 5. Effects of accumulation of doses of ATP on the contractions of guinea-pig bladder produced by nerve stimulation and ATP. The nerve mediated responses produced by single electrical pulses delivered at 2 min intervals are marked by a white dot above the response. At the open triangles, 60 s after a pulse, ACh (10^{-7} g/ml) was added to the bath and removed after 30 s by an overflow washout for 10 seconds. At the closed triangles, 60 s after a pulse, ATP (A) was added to the organ bath and was not washed out. Repetitions of three different doses are shown. Note that the response to each successive dose of ATP at the two higher dose levels decreases markedly. The nerve mediated contractions are depressed during desensitization with 10^{-4} g/ml of ATP. The response to ACh is also depressed. Numbers at bottom of the figure indicate the dose added to the organ bath in μg/ml. Time marker, 2 minutes.

an inhibition. That the blocking action of quinidine was not simply an antispasmodic effect was clear, because acetylcholine still produced a contraction, although of reduced amplitude. It is therefore clear that the abolition of the nerve mediated response by quinidine is not due to antagonism of acetylcholine. The block of the nerve mediated contractions can be more readily explained by assuming that the nerves act by releasing adenosine triphosphate as a transmitter substance. Adenosine triphosphate would have been more clearly implicated in nerve mediated responses if they, like the responses to adenosine triphosphate, had been reversed by quinidine. However, a nerve mediated inhibition was not seen in these experiments. The blockade of the nerve mediated response is not likely to be the result of a local anaesthetic action of quinidine, since increasing the stimulation voltage did not restore the responses.

The inhibitory action of adenosine triphosphate on the rabbit ileum displays tachyphylaxis (Mihich, Clarke & Philips, 1954). Also, Ambache & Zar (1970) have shown that tachyphylaxis to adenosine triphosphate is developed by the guinea-pig urinary bladder. In the present experiments it was only when the adenosine triphosphate was left in the bath that a marked reduction in the amplitudes of the responses to successive doses resulted. Urinary bladders desensitized in this way to high doses of adenosine triphosphate (10^{-4} g/ml) exhibited reduced contractions to both a single pulse or tetanic nerve stimulation, but also depression of the contractions produced by histamine and acetylcholine. Thus, although these results are consistent with adenosine triphosphate participating in transmission to the bladder, they fail to prove it.

Ambache & Zar (1970) argued against a role of adenosine triphosphate in excitatory transmission, on the grounds that the contractions produced by single electrical pulses were not depressed during the tachyphylaxis and desensitization to adenosine triphosphate. We were unable to repeat their results. When doses of adenosine triphosphate were applied repeatedly to the tissue and washed out of the bath, as practised by these workers, no significant tachyphylaxis to adenosine triphosphate ever developed. In contrast, in a second series of experiments, desensitization was produced to adenosine triphosphate (10^{-5} g/ml) when it was allowed to accumulate in the organ bath but there was little concurrent reduction of responses to single electrical pulses. It remains to explain why the nerve mediated twitch was not depressed during tachyphylaxis or desensitization to the lower doses of adenosine triphosphate, assuming the transmitter is in fact adenosine triphosphate. It may be that the adenosine triphosphate released from the nerves acts upon receptors which are not accessible to exogenous adenosine triphosphate. However, the absence of morphological evidence for a barrier around synaptic areas in the bladder has already been pointed out (Dumsday, 1971). Alternatively the situation may be compared to the failure of adrenoceptor blocking drugs to block adrenergic nerve transmission to vascular smooth muscle. α-Adrenoceptor blocking drugs fail to prevent responses to stimulation of what are clearly adrenergic nerves while blocking the responses to equipotent doses of exogenously applied catecholamines (Nickerson, 1949). The differential sensitivity to adrenoceptor blockade is evident for both non-competitive and competitive blocking drugs. The reason for the difference in susceptibility to blockade of the two stimuli is not yet known. It is clear then, that the persistence of the nerve mediated ' twitch ' during the depression of the response to the (formerly) ' twitch-matching dose ' of adenosine triphosphate added to the

organ bath does not provide evidence against a role of adenosine triphosphate in mediating the excitatory response to nerve stimulation. It may be that a more rapid increase in concentration of nerve released adenosine triphosphate and/or a higher concentration of adenosine triphosphate at the receptors will be capable of overcoming the tachyphylaxis and desensitization to exogenously applied adenosine triphosphate.

Apparently the strongest evidence against the suggestion that adenosine triphosphate is acting as an excitatory transmitter substance is that adenosine triphosphate, in concentrations as high as 1 mM, caused contractions smaller than those induced by tetanic nerve stimulation. Before reaching the receptors, however, the concentration of applied adenosine triphosphate would probably be reduced further by degradation and uptake than would the concentration of adenosine triphosphate neurogenically released adjacent to receptor sites. Nevertheless it may be argued that sufficient adenosine triphosphate was added to overcome the inactivation to which an externally applied drug is subjected.

This study has revealed some interesting facts about the receptors in the urinary bladder for purine-based compounds. In mammalian gut both adenosine triphosphate and adenosine produce inhibition, although adenosine is 100-fold less active. In the bladder, however, these substances have differential effects, that is adenosine triphosphate causes excitation but adenosine causes inhibition. An excitation was produced by all purine compounds tested which contained a pyrophosphate bond. However, sodium pyrophosphate alone had no effect on the bladder, indicating that both a purine compound and a pyrophosphate group are necessary for excitatory activity. All of the adenosine-based compounds, but not inosine or guanosine, were able to cause an inhibition of the urinary bladder. With adenosine triphosphate and adenosine diphosphate an inhibition was only seen after the excitatory effects of these compounds were prevented by quinidine.

The authors wish to thank Dr. G. D. Campbell for detailed discussions of the manuscript. This work was supported by grants from the Australian Research Grants Committee and the National Heart Foundation of Australia.

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(*Received August* 28, 1971)