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The actions of ketotifen on intestinal smooth muscles

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Abstract

Ketotifen is a tricyclic drug with a wide spectrum of pharmacological effects. We studied the actions of ketotifen on the mechanical activity of isolated segments of guinea-pig ileum, guinea-pig colon and mouse colon. In the guinea-pig ileum ketotifen induced small contractions and inhibited the contractions induced by carbachol and by electric field stimulation. Responses to bradykinin (in the absence or in the presence of atropine 1 μM) were similarly inhibited by ketotifen, with an IC_{50} of 23 μM. In the guinea-pig colon ketotifen evoked non-cholinergic contractions with a pD_2 of 4.5, but still it inhibited responses to bradykinin (IC_{50} = 75 μM). Ketotifen relaxed the unstimulated mouse colon with a pD_2 of 4.2. This effect persisted in the presence of propranolol and phentolamine (each 10 μM). Incubation of the mouse colon with the nitric oxide synthase inhibitors N^G-nitro-L-Arginine methyl ester hydrochloride (L-NAME), or L-N^G-nitro-arginine (L-NNA) (100–500 μM) did not alter the inhibitory action of ketotifen. The histamine H_1 receptor antagonist chlorpheniramine (5 μM) or the nerve blocker tetrodotoxin (1 μM) did not alter the inhibitory effects of ketotifen. It is concluded that the actions of ketotifen are mediated by a non-cholinergic, non-histaminergic mechanisms.

Keywords: Ketotifen; Intestine; Noradrenaline; Nitric oxide (NO); Smooth muscle

1. Introduction

Ketotifen is a tricyclic compound used mainly as an anti-asthmatic drug (Grant et al., 1990). Recently it has been shown that ketotifen has an anti-inflammatory action in the stomach and large intestine (Pothoulakis et al., 1993). The mechanisms of action of ketotifen are not entirely clear, and its anti-asthmatic effect has been ascribed to its being a mast cell stabilizer. Ketotifen was reported to act as an antimuscarinic drug in the trachea (Polson et al., 1982), but non-cholinergic inhibition by this drug has also been described (Verleden et al., 1994). Eltze et al. (1992) studied the effects of ketotifen on the contractile activity of isolated smooth muscle preparations and concluded that it acted as a non-selective muscarinic blocker. This lack of selectivity, and the fact that ketotifen has a wide spectrum of apparently unrelated effects prompted us to reexamine its actions on several intestinal preparations.

2. Materials and methods

The experiments were done on male guinea-pigs (300–500 g), and on Balb/C mice of either sex (25–30 g). Guinea-pigs were stunned and bled, and mice were stunned and killed by cervical dislocation. The following intestinal segments were removed: non-terminal ileum (10–15 cm from the cecum) and distal colon from guinea-pigs and distal colon from mice. Two to three tissues were obtained from each animal. The tissues were placed in cold Krebs solution of the following composition (mM): NaCl 120.7; KCl 5.9; $NaHCO_3$ 14.4; $MgSO_4$ 1.2; NaH_2PO_4 1.5; $CaCl_2$ 2.5 and glucose 11.5. Longitudinal intestinal segments (1–1.5 cm long) were suspended under a constant tension of 1 g in a 10 ml organ bath containing Krebs solution. The solution was kept at 37°C and was bubbled with a mixture of 95% O_2 and 5% CO_2. Force was measured with an isometric transducer (Gould UC2) and was recorded on a Gould model 2200S chart recorder. Electrical stimulations were delivered by two platinum ring electrodes from Grass S88 stimulator, pulse duration was 0.5 ms and supramaximal voltage (50 V) was used. Responses to agonists were recorded for 4–10 min. Antagonists were added to the bath at least 10 min before testing their effects. Periods of 15–30 min were allowed between sub-

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sequent measurements to allow full recovery from drug actions. To determine whether drug effects depended on the muscle tone we measured responses at resting tones in the range 0.2–1.5 g. These tones were achieved by stretching the preparations until the desired values were reached.

Drugs: ketotifen fumarate, carbamylcholine chloride (carbachol), bradykinin acetate, substance P, propranolol hydrochloride, histamine hydrochloride, hexamethonium bromide, isoprenaline hydrochloride, phentolamine hydrochloride, tetrodotoxin, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), L-N^G-nitro-arginine (L-NNA) were purchased from Sigma.

In the cases where ketotifen acted as an agonist, its activity is indicated by pD_2 ($-\log$ of the concentration that produced 50% of the maximum effect). In cases where it acted as an antagonist, this activity is indicated by IC_{50} (the concentration of ketotifen that produced a 50% reduction in the contractions evoked by an agonist). The results are presented as means ± S.E.M. Statistical significance was evaluated by Student's *t*-test for paired or unpaired values where appropriate. Significance was set at $P < 0.05$ level.

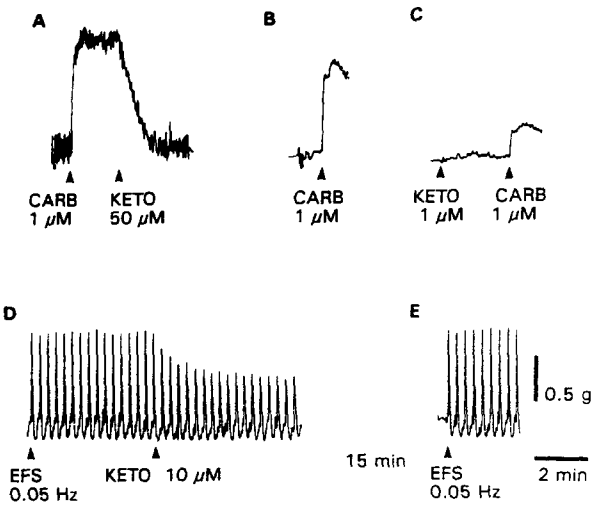

Fig. 1. The inhibitory effects of ketotifen on the isolated guinea-pig ileum. (A) Ketotifen (KETO) completely relaxed the tissue precontraced with carbachol (CARB). (B) A control response to carbachol. (C) Ketotifen was applied before carbachol and greatly reduced the response. (D) Responses to electric field stimulations (EFS) were reduced by ketotifen. (E) Reversal of the ketotifen effect 15 min after washout. The results are from three different preparations. Each of these results was obtained in eight preparations.

3. Results

3.1. Guinea-pig ileum

Carbachol (1 μM) caused a tonic contraction of the longitudinal muscle of the guinea-pig ileum, which was abolished by ketotifen (Fig. 1A). Similarly, when ketotifen was applied to the bath prior to the addition of carbachol, it inhibited carbachol's effect (Fig. 1B,C). Ketotifen also inhibited contractions induced by electric field stimulation (Fig. 1D,E). These contractions were largely due to acetylcholine release as they were blocked by 1 μM atropine (data not shown). These effects of ketotifen were largely reversible after 15–30 min of wash (Fig. 1E).

These results suggested that ketotifen inhibited the responses to carbachol or to electrical stimulation by blocking muscarinic acetylcholine receptors, as proposed by Eltze et al. (1992). To test this point we measured the effect of ketotifen on the contractile response to bradykinin. In control experiments we found that the response to bradykinin was not altered by atropine (1 μM, $N = 6$, $P > 0.5$, paired *t*-test) or by hexamethonium (0.2 mM, $N = 7$, $P > 0.1$), and it can be concluded that bradykinin acted via a non-cholinergic mechanism. Fig. 2 shows that the contractions induced by bradykinin (20 nM) in the presence of atropine (1 μM) were strongly inhibited by ketotifen; the IC_{50} being 23 μM. This inhibitory influence of ketotifen was also observed in the absence of atropine (for ketotifen concentrations of 10 and 50 μM, $N = 6$, $P > 0.1$, paired *t*-test). These results indicated that ketotifen acted by a non-cholinergic mechanism.

At a resting tone of 1 g ketotifen alone in the concentration range 1–10 μM had no effect on the muscle tone ($N = 10$, Fig. 1C). At concentrations of 50 μM and higher ketotifen evoked small, brief contractions that were not blocked by atropine (1 μM). This pattern was not altered when resting tone was varied in the range 0.2–1.5 g ($N = 9$).

3.2. Guinea-pig colon

In the longitudinal muscle of the guinea-pig colon ketotifen alone induced contractions at all the resting tones in the range 0.2–1.5 g ($N = 6$), see Fig. 3. This effect was studied in detail on 20 preparations at a resting tone of 1 g and was found to be concentration-dependent with pD_2 of

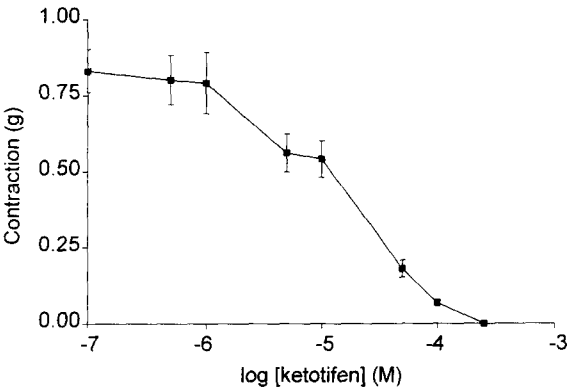

Fig. 2. The effect of ketotifen on responses of the guinea-pig ileum to bradykinin (20 nM), in this and in all the following experiments the measurements were done in the presence of atropinne (1 μM). Ketotifen was added 3 min before the application of bradykinin to the bath, and the maximum response to bradykinin was measured ($N = 9$).

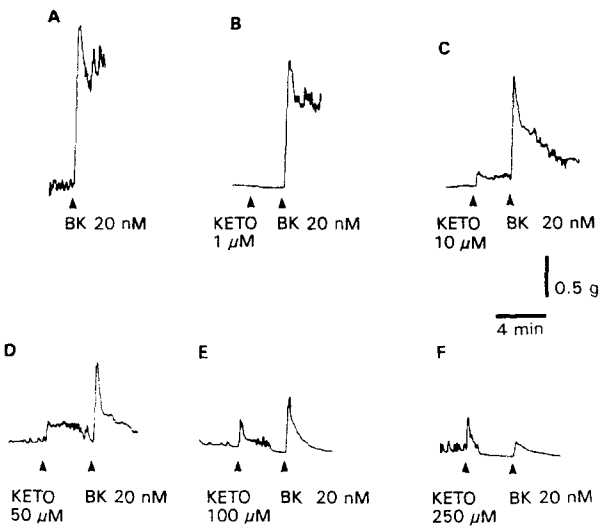

Fig. 3. The effect of ketotifen on bradykinin-induced contractions of the guinea-pig colon. Ketotifen was added to the bath 3 min before the applications of bradykinin (20 nM). Note that in addition to its direct contractile action, ketotifen inhibited the responses to bradykinin. These results were obtained in 20 preparations.

4.5. These contractions were not altered by atropine (1 μM). The ketotifen-induced contractions increased when the tone was raised; for example, at 1.5 g the contraction was 1.9 ± 0.31 greater than at 0.5 g ($P < 0.05$, paired t-test, $N = 6$). To determine whether ketotifen had an inhibitory action in this tissue we examined the responses to bradykinin in the presence of ketotifen. As shown in Fig. 3, ketotifen contracted the muscle but still antagonized the responses to bradykinin. The concentration dependence of this effect is shown in Fig. 4 ($IC_{50} = 75$ μM). All these measurements were done in the presence of atropine (1 μM).

3.3. Mouse colon

At the resting tone used for most of the experiments (1 g), ketotifen evoked a biphasic response in 25 of 47

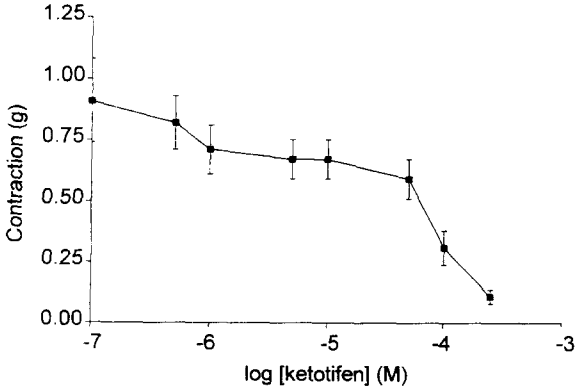

Fig. 4. The concentration dependence of the inhibitory effect of ketotifen on the contractions induced by bradykinin (20 nM) on the guinea-pig colon. Ketotifen was added to the bath 3 min before the applications of bradykinin ($N = 10$).

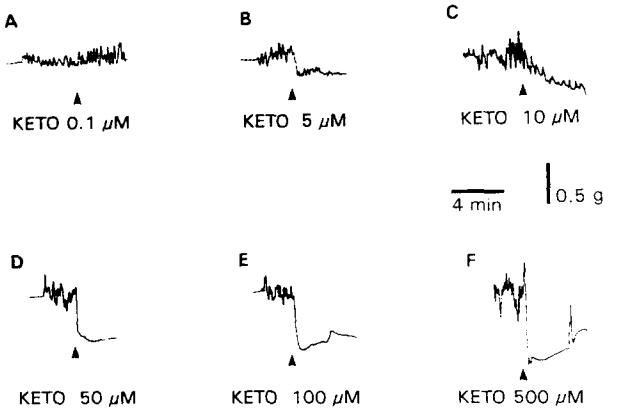

Fig. 5. Relaxations evoked by ketotifen in the mouse colon. This effect was concentration-dependent ($pD_2 = 4.2$, $N = 24$).

preparations, consisting of a brief, transient contraction followed by a prominent and long-lasting relaxation. In the other 22 cases only prolonged relaxation was observed (Fig. 5). In all cases the relaxation persisted as long as the tissue was in contact with the drug (followed for 10–15 min) and its amplitude was concentration-dependent with $pD_2 = 4.2$ (measured in 24 preparations). All these observations were made in the presence of atropine (1 μM). The effects of ketotifen were examined on the tissue when the resting tone was maintained at values between 0.2 and 1.5 g. In all cases ($N = 14$) the transient contraction was not significantly influenced by the resting tone, but the amplitude of the relaxation increased when the tone was raised.

Unlike in the guinea-pig small and large intestines, in most cases bradykinin did not cause contraction in the mouse colon. To examine the effects of ketotifen on non-cholinergic contractions we used substance P in the presence of atropine (1 μM). Ketotifen at 10 μM ($N = 8$) and 50 μM ($N = 5$) reduced contractions evoked by substance P (0.1 μM) by 23.0 and 88.1%, respectively ($P < 0.005$, compared with control, paired t-test).

3.4. What is the mechanism of ketotifen actions?

These actions of ketotifen could be due to the release of an inhibitory substance from nerves. To test this possibility we incubated the guinea-pig ileum and colon and the mouse colon in tetrodotoxin (1 μM, $N = 6$ for each type of tissue). We found that tetrodotoxin did not alter the inhibitory effects of ketotifen described above for these three preparations. In the guinea-pig colon the ketotifen-induced contractions were not affected by tetrodotoxin ($N = 6$).

The best known action of ketotifen is to stabilize mast cells (Grant et al., 1990) and it could be proposed that its inhibitory actions were due to inhibiting histamine release from these cells (assuming that histamine contracts the muscle), or to inhibiting histamine's action on the muscle. To test these possibilities we first examined the effect of

histamine. Histamine contracted the guinea-pig ileum at concentrations of 1 and 10 μM ($N = 4$), and the guinea-pig colon at 5 and 20 μM ($N = 4$). These contractions were blocked by the histamine H_1 receptor antagonist chlorpheniramine (5 μM). Chlorpheniramine (5 μM) itself did not have a consistent effect on muscle tone in the guinea-pig ileum ($N = 4$) or colon ($N = 4$), indicating that there was no tonic release of histamine. Also, chlorpheniramine (1–10 μM) did not block ketotifen-induced contractions in the guinea-pig ileum ($N = 4$, $P > 0.1$) and guinea-pig colon ($N = 4$, $P > 0.6$). In the mouse colon histamine did not elicit a response even at a very high concentration (20 μM, $N = 6$). Chlorpheniramine (5 μM, $N = 6$) contracted the mouse colon, apparently via a histamine-independent mechanism. Chlorpheniramine (1–10 μM) did not affect the ketotifen response on the mouse colon ($N = 6$, $P > 0.3$). Thus it appeared that ketotifen's actions in the three tissues were not related to histamine release.

The mouse colon was found to be strongly relaxed by β- adrenoceptor agonists (Fontaine et al., 1984; Hanani, 1990), thus the inhibitory effects of ketotifen could be explained by a direct β-adrenoceptor-mediated effect. The following experiment was designed to test this idea. In control measurements we observed the inhibitory influence of the β-adrenoceptor agonist isoprenaline, which was largely blocked after adding propranolol and phentolamine (each 10 μM). This blockade was reversed on washing. In the same preparation ketotifen evoked a contraction followed by relaxation. The adrenergic blockers did not alter the inhibitory effect of ketotifen. These results were obtained in 13 preparations.

Nitric oxide (NO) is another agent that potently relaxes intestinal smooth muscle (Stark and Szurszewski, 1992; Sanders and Ward, 1992). We next tested the hypothesis that NO may be released in the mouse colon by ketotifen. Electrical field stimulation induced relaxation followed by contraction. L-NAME, an inhibitor of NO synthesis, was added at concentrations of 100 or 500 μM and greatly reduced the inhibitory phase. Under these conditions ketotifen relaxed the muscle as in the control ($N = 12$). Similar results were obtained with another NO-synthase inhibitor, L-NNA (100 or 500 μM, $N = 10$).

4. Discussion

The principal conclusion from the results above is that ketotifen acted on intestinal muscle via non-cholinergic mechanisms. The main evidence for this conclusion is that ketotifen exerted its effects in the presence of atropine at a concentration that completely blocked muscarinic acetylcholine responses. The finding that these actions were not blocked by tetrodotoxin indicated that ketotifen did not release inhibitory substances from nerves but acted directly on the muscle.

The mechanisms that mediate the effects of ketotifen on intestinal smooth muscle are unclear, and the presence of both inhibitory and excitatory influences is a further complication. In the guinea-pig ileum and colon ketotifen alone evoked transient contractions but it still inhibited contractions induced by carbachol or bradykinin. The ketotifen-induced contractions were not mediated by acetylcholine receptors as they were not blocked by atropine. Ketotifen inhibited muscarinic acetylcholine responses evoked by electric field stimulation and by the application of carbachol. This inhibitory effect was apparently not mediated by acetylcholine receptors because non-cholinergic responses to bradykinin or to substance P were also blocked by ketotifen. However, a minor cholinergic influence of the drug cannot be excluded.

Ketotifen is known to be a mast cell stabilizer (Grant et al., 1990) and it can be suggested that its inhibitory actions may be related to this effect. However, we found that in the guinea-pig ileum and colon the histamine H_1 receptor antagonist chlorpheniramine did not affect the resting tone, indicating the absence of tonic release of histamine. Moreover, chlorpheniramine did not alter the inhibitory action of ketotifen in the mouse colon, and it can be concluded the actions of ketotifen were not mediated by histamine.

Kamikawa (1989) and Verleden et al. (1994) reported that in the guinea-pig isolated trachea ketotifen inhibited non-cholinergic contractions, apparently by inhibiting transmitter release from nerves. Our experiments with tetrodotoxin indicated that in the intestine ketotifen acted directly on the muscle rather than prejunctionally. We tested the possibility that ketotifen relaxed the mouse colon by activating β-adrenoceptors, which are inhibitory in this preparation (Fontaine et al., 1984; Hanani, 1990), or by blocking the synthesis of NO, which is a potent relaxing agent in intestinal muscles (Stark and Szurszewski, 1992; Sanders and Ward, 1992). In both cases the actions of ketotifen were not blocked. Thus, in the intestine, ketotifen apparently acted by activating a yet unidentified receptor or by modifying a post-receptor mechanism such as the metabolism of second messengers (e.g. Ca^{2+} or cyclic nucleotides). Castillo et al. (1990) showed that ketotifen inhibited phosphodiesterase activity, thereby allowing the accumulation of cyclic AMP. Whatever is the precise pathway, it is likely that ketotifen inhibited muscle contraction by some generalized mechanism and not via the blockade of acetylcholine receptors (see Eltze et al., 1992). This conclusion is in accord with previous studies that showed that ketotifen could inhibit contractile responses to agents other than acetylcholine: histamine (Wilhelms, 1987) or prostaglandin $F_{2\alpha}$ (Alvarez and Arruzazabala, 1983). However, the site of action has not been determined in these studies. Our conclusions may be relevant for other tricyclic compounds. Our preliminary (unpublished) results suggested that the tricyclic compound cyproheptadine also acts on intestinal muscle via a non-cholinergic mechanism. Thus, the results on ketotifen may contribute to the understanding of the pharmacology of this important family of drugs.

Our results may have clinical relevance. Therapeutic plasma concentrations of ketotifen in humans are in the range of 1–4 μg/ml (Jeffreys and Volans, 1981), which corresponds to about 2.5–10 μM. In our experiments the threshold for ketotifen effects was about 1 μM. Thus the direct effects on muscle described above may be relevant to clinical situations. We have shown that ketotifen can relax smooth muscle directly or inhibit the actions of excitatory agents, and it can therefore be suggested that part of its therapeutic action on the respiratory tract may be mediated by a similar effect.

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