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The anti-oestrogen tamoxifen is a calcium antagonist in perfused rat mesentery

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Summary. The anti-oestrogen tamoxifen in the range 10^{-7} – 10^{-5} *M* induced concentration-related inhibition of potassium-stimulated vasospasm in the isolated perfused rat mesentery vascular bed. In contrast, responses to noradrenaline did not fall below control levels until the tamoxifen concentration exceeded 4×10^{-6} *M*. Recovery of the potassium-stimulated responses was also concentration-related. The most likely explanation is that while tamoxifen can obstruct the entry of extracellular calcium, it is unable to prevent the intracellular release of the ion by noradrenaline.

Introduction

The anti-oestrogen tamoxifen (TAM) has been widely used in clinical trials for over 10 years in the treatment of breast cancer [3, 11, 12]. It has been shown to bind to the oestrogen receptor with about 1% of the affinity of oestradiol, and it is generally accepted that competition for the oestrogen receptor is of major importance in its action on breast tumours which possess oestrogen receptors. However, there are still some anomalies in the reports of its mode of action.

Thus, TAM has been shown to be effective in some estrogen receptor-negative breast tumours, and even in oestrogen-receptor-positive tumours the therapeutic concentration required (about 10^{-6} *M*) is at least an order of magnitude greater than that theoretically needed to saturate all oestrogen receptors [3, 13], even allowing for the relatively low binding affinity. There have also been reports of cases where oestrogen-dependent tumours have not responded to tamoxifen [3, 11, 12].

We have shown that in vitro TAM will inhibit contractility of those kinds of smooth muscle cells which depend directly on extracellular calcium (Ca^{2+}) entry for contraction (e.g. smooth muscles of the uterus and gastrointestinal tract), while exerting little or no inhibition of contractions to organic spasmogens on isolated aorta [6, 7] or on trachea or skeletal nerve/muscle preparations, which can utilise intracellular stores of Ca^{2+} (submitted for publication) Morris (1985) independently obtained similar results with intestinal smooth muscle [10].

It therefore seemed possible that this selectivity could be due to an action on Ca^{2+} transport or metabolism. In support of this hypothesis we have demonstrated that TAM inhibits potassium (K^+)-induced contractions of isolated aorta and uterus in a dose-related manner [6]. In other, unpublished studies, TAM had little effect on noradrenaline (NA) responses of the aorta. The K^+ effect is generally accepted as due largely to voltage-stimulated increased entry of extracellular Ca^{2+}, while much of the NA response of the aorta has been shown to involve release of intracellular Ca^{2+} [2, 8, 9]. We can speculate that the Ca^{2+}-blocking action could be independent of the oestrogen receptor or one of the effects of TAM on the receptor in the intact individual.

This paper presents data which support the concept of an action by TAM on Ca^{2+} entry rather than on intracellular Ca^{2+} release. The preparation used was the vascular bed supplied by the inferior mesenteric artery of the adult rat, which responds both to NA and to increased K^+ concentration by increased resistance to flow.

Materials and methods

Adult female Sprague-Dawley rats weighing 200–230 g and ovariectomised at least 1 month previously were given a single dose of oestradiol valerate in sesame oil (40 μg/kg body wt i.m.). The viscera were exposed 3–5 days later by a mid-line ventral abdominal incision made during ether anaesthesia. All uteri were large and swollen with fluid, confirming uptake of the hormone.

The inferior mesenteric artery was cannulated at the aortic join, and 1.0 ml heparin sodium solution in saline (2.5 mg/ml) was infused immediately. The mesenteric distribution bed was separated from all visceral organs and the gut as rapidly as possible by cutting close to the intestinal border of the mesentery. The cannula was then connected to a peristaltic perfusion pump with a flow rate of 3 ml/min and 'pulse' rate of 100/min. The control perfusate used was a modified Krebs medium containing (m*M*/l^{-1}): NaCl, 122; KCl, 4.3; $CaCl_2$, 1.25; $MgCl_2$, 1.0; NaH_2PO_4, 0.84; $C_6H_{12}O_6$, 5.6; $NaHCO_3$, 25, freshly prepared each day from stock solutions, except for the glucose and bicarbonate, which were added as solids. The perfusate was bubbled constantly with 5% CO_2, 95% O_2, and warmed to 37° C. The tissue was suspended from its

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cannula in a jacketed organ-bath maintained at 37° C and open at the bottom to allow free escape of the expended perfusate.

Pressure was recorded on a Devices physiograph via a Gould pressure transducer connected to the perfusion cannula just before it entered the preparation. Four preparations were mounted in parallel for each experiment and left to stabilise for at least 30 min. When the pressure was steady, at about 25–35 mmHg, the perfusate was changed to a 'high K^+' solution, which was identical to the control solution except that it contained 86 m*M* KCl, 40 m*M* NaCl. The spasmogenic effect was recorded for approximately 5 min and then the control perfusate was resumed; pressure returned to basal levels within 1–2 min. After about 15 min, NA solution (Arterenol hydrochloride, Sigma, Mo, USA, 10 μg/ml in saline) was added to the perfusate just before the cannula from a syringe-type slow infusion apparatus which delivered 1 μg ml^{-1} min^{-1}.

Pressure changes that developed in response to high K^+ or NA were measured on the physiograph record by manually integrating the area under the curve during the first 4 min of each response, and were expressed as grams of tension per minute. The height of the 'initial peak' in the K^+ response was not significantly different from that of the steady state part of the response or the integrated response.

When two or three successive K^+ and NA responses about 15 min apart showed no change (initial controls), two of the preparations were changed to a perfusate containing a selected concentration of TAM and two were maintained on control perfusate. The testing of all four continued as before, alternating K^+ and NA. The high K^+ perfusate for TAM-treated preparations also contained TAM at the selected concentration.

When, after the addition of TAM, three successive responses to high K^+ showed no further change (usually within 3 h), control perfusate was restored to the tissues and recovery followed for 1 h, spasmogen testing continuing as before.

Dilute TAM solutions are sensitive to ultraviolet radiation, so all containers and tubing were wrapped in aluminium foil to minimize decomposition of the drug.

Results

Figure 1 shows log dose-response curves for the action of TAM on mesenteric bed responses to high K^+ or NA, together with control means. K^+ contractures were inhibited in a dose-related manner, between about 10^{-7} *M* TAM and 4×10^{-6} *M* TAM, with 50% of initial control response at 3×10^{-7} *M* TAM. Responses to NA were only significantly reduced below controls by 10^{-5} *M* TAM. Control preparations showed steady increases in response to both spasmogens with time, responses at 3 h being about 120% of initial responses. The responses to NA in concentrations of TAM up to 5×10^{-7} *M* also showed steady increases with time, but not significantly greater than those of control preparations after 3 h. With higher TAM concentrations the NA responses were progressively smaller.

While individual responses to NA were not significantly different from control values up to 4×10^{-6} *M* TAM, the highly significant slope of the dotted line in Fig. 1, which is a linear regression plotted between 1.25×10^{-7} *M* and 4×10^{-6} *M* TAM (ANOVAR: $F = 10.46$; 1 vs 18 *df*; $P < 0.01$), suggested that the effects of TAM on this spasmogen were also dose-related. This might be due to TAM inhibition of the slow inward movement of Ca^{2+}, which is said to be the cause of the steady state portion of the NA response [8, 9].

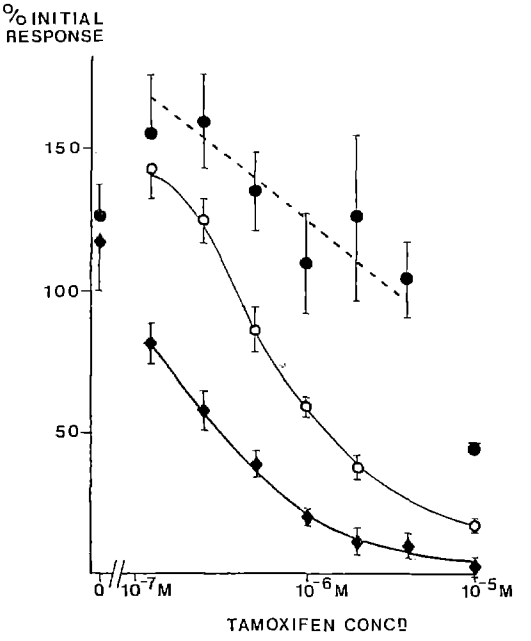

Fig. 1. Log dose-response for tamoxifen effect on K^+- (◆) and NA (●)-stimulated resistance to flow of the perfused rat mesentery. Also included is recovery (○) of K^+ response in control perfusate 1 h after removal of TAM. *Points* at zero concentration represent responses of controls after 3 h. All *points* represent means ± SEM of responses at equilibrium expressed as % of initial control means at equilibrium. ($n = 4$ for each *point*)

The final recovery levels reached for K^+ responses in 1 h after the TAM solution was replaced by the control perfusate are also shown in Fig. 1, expressed as percen-

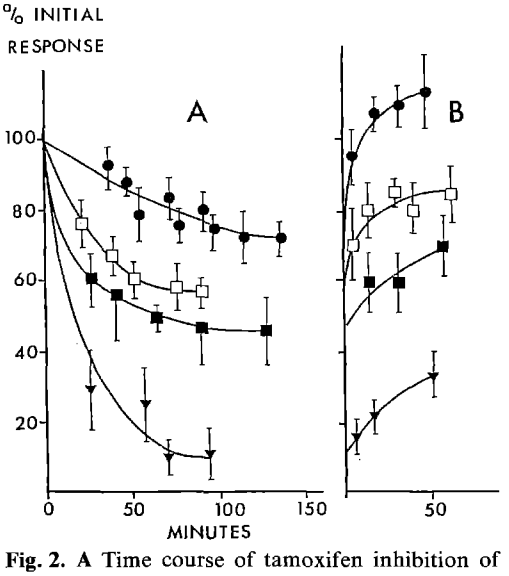

Fig. 2. **A** Time course of tamoxifen inhibition of vasospasm induced by high K^+ perfusate at 4 concentrations; **B** subsequent recovery on return to normal Krebs perfusate. ●, 1.25×10^{-7} *M*; □ 2.5×10^{-7} *M*; ■, 5×10^{-7} *M*; ▼, 2×10^{-6} *M*. ($n = 4$ for each *point*)

tages of initial control values. In Fig. 2 the time course of inhibition and recovery illustrates that both are clearly dose-related.

Discussion

It is clear that TAM induces reversible, dose-related inhibition of the vascular response of rat mesentery to high K^+, but fails to inhibit responses to NA below initial control levels until much higher concentrations are perfused. The IC_{50} for high K^+ of about 3×10^{-7} *M* TAM shows that this preparation is about twice as sensitive to TAM as the isolated aorta or myometrium and the recovery from TAM within 1 h is greater than that shown by rat myometrium or aorta at all dose levels [7]. The much weaker inhibition of NA responses and the reversibility of the inhibition of responses to high K^+ demonstrates that the inhibition is not due to some non-specific toxicity.

The simple explanation for these results is that TAM obstructs K^+-stimulated Ca^{2+} entry into the smooth muscle, but is unable to prevent the release of intracellular Ca^{2+} by NA. However, in our earlier study with isolated aorta and myometrium [6], we were unable to demonstrate significant reduction of potassium-stimulated $^{45}Ca^{2+}$ uptake by TAM.

It is possible that TAM obstructs Ca^{2+} transport in the cytoplasm, but only when the ion is of extracellular origin and not when it is released from intracellular stores. Lam [4] has shown that TAM blocks Ca^{2+} uptake by calmodulin in vitro by interference with phosphodiesterease. NA might bypass this step when releasing intracellular Ca^{2+}. We have recently demonstrated that TAM can reduce prostacyclin and thromboxane output from this preparation at a concentration used therapeutically (10^{-6} *M*) (to be published), so another possible explanation for the reduced vascular resistance of this preparation could be reduced thromboxane vasopressor activity, especially if this is also due to an effect on Ca^{2+} entry, rather than on intracellular release. The tendency for both control and NA perfused preparations to exhibit slightly increased vascular resistance after 3 h perfusion at the lower TAM concentrations (Fig. 1) is not easy to explain, but we might speculate that it could be due to washing out of the endothelial relaxing factor, or interfering with its synthesis. Neither isolated stripped aorta nor myometrium smooth muscles showed increased contractility during exposure to the vehicle or the drug.

High plasma Ca^{2+} levels might account for the limited effectiveness of TAM in some women if the competitive antagonism demonstrated with isolated tissues [6] also occurs in vivo. This possibility is supported by the proposal [1] that increased cell membrane permeability to Ca^{2+} ions is a primary factor in carcinogenesis. High intracellular Ca^{2+} is suggested as a cause of mitochondrial and nuclear damage leading to malfunction of both bioenergetic and genetic mechanisms. Ca^{2+} antagonism by TAM may tend to reduce this damage.

There are also well substantiated reports that when TAM is first administered it induces a temporary hypercalcaemia, which may be the result of reduced uptake of the ion by various tissues, which is subsequently compensated by increased excretion [5, 11].

In view of these and previous findings [5], it appears that it would be worthwhile to investigate the plasma Ca^{2+} levels of TAM-treated patients. Whether Ca^{2+} antagonism is directly involved in mammary tumour regression by TAM or whether it is a modifying factor for some other action on the neoplasms is under investigation.

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