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Calcium antagonism by the antioestrogen tamoxifen*

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Summary. Tamoxifen, an oestrogen receptor antagonist and an effective treatment for breast carcinoma, has recently been shown to possess spasmolytic activity in smooth muscle. Tamoxifen in vitro inhibited the contraction of smooth muscle from rat myometrium and aorta produced by exogenous calcium. At the same concentration tamoxifen did not affect the uptake of calcium into the muscle. The importance of calcium in cell proliferation suggests that some of the unexplained antitumour activity of the oestrogen antagonists may be accounted for by intracellular calcium antagonism.

Introduction

Tamoxifen is an effective treatment for breast cancer [6, 14], its activity being explained by antioestrogenicity brought about by competition for the steroid receptor [12]. In vitro low concentrations ($< 10^{-6}$ *M*) of tamoxifen inhibit cell proliferation of breast tumour cells by interaction with oestrogen receptors, but cytotoxicity at high concentrations ($> 10^{-6}$ *M*) appears unrelated to receptors or the antioestrogen binding site [2, 17, 18, 20]. Serum and tissue tamoxifen concentrations in patients often exceed 1×10^{-6} *M* during treatment [6]. We have recently reported that in vitro high concentrations of antioestrogens inhibit the contractions of myometrial and ileal smooth muscle [15, 16]. Here we demonstrate in isolated rat myometria and to a lesser extent in aorta that high concentrations of tamoxifen antagonise calcium-induced contractions, and that this is not reflected by changes in $^{45}Ca^{2+}$ uptake. As calcium is essential for cell division [8], intracellular calcium antagonism is probably an important contributor to the antitumour activity of tamoxifen.

Materials and methods

Sprague-Dawley female rats weighing 100–200 g were ovariectomized, and 3–7 weeks later dosed with 40 μg/kg body weight oestradiol propionate in arachis oil i. m. and killed 3–5 days later. The endometrium was stripped from the myometrium and the muscle cut into pieces 2–3 mm wide and 20–30 mm long. The thoracic aorta was opened longitudinally, and the endothelium was removed by gentle rubbing with a cotton tip and then cut into pieces about 4 mm in length. The tissues were suspended on force transducers in isolated organ baths under resting loads of 1 g bathed in a modified MOPS [3-(*N*-morpholine)propane sulphonic acid]-buffered Krebs solution at 37 °C (medium a, see below). This physiological salt solution was used to ensure comparability with the parallel isotope studies, as this protocol included $LaCl_3$ in the rinse medium, which was incompatible with ordinary bicarbonate-Krebs. Contractions were recorded isometrically using a Rikadenki R02 electronic recorder. After about 20 min the vehicle (0.25% ethanol) was added to the baths and concentrations maintained throughout. Repeated exchange of the baths for normal Ca^{2+} depolarising MOPS buffer (medium b) was performed as described in the text. When consistent responses had been obtained tamoxifen or vehicle was added to the bath and equilibration of responses achieved once more. At this time the solution in the tissue bath was changed to Ca^{2+}-free depolarising buffer (medium c). Once relaxed the $CaCl_2$ was added to the tissue bath in a cumulative manner at 5- to 10-min intervals, which produced graded tension development.

A modification of the lanthanum method of van Breeman et al. [4] was used to investigate the effect of tamoxifen upon the uptake of calcium into the tissues. Rats were treated and tissues prepared as described above. Tissues were impaled on syringe needles attached to a manifold, which facilitated rapid transfer and also served to supply oxygen (100%). Tissues were suspended in medium a at 36 °C and allowed to equilibrate for 20–30 min. Tissues were then transferred to tubes containing the same medium but with tamoxifen or 0.25% ethanol vehicle present. After 3½ h the tissues were transferred into medium a containing in addition 0.5 μCi $^{45}Ca^{2+}$ per ml (10 mCi/mg Amersham International plc, UK) for 5 min. Tissues were then transferred either to normal medium (medium a) or to K^+ depolarising medium (medium b) with or without tamoxifen also containing $^{45}Ca^{2+}$ for 10 min. At the end of this incubation the tissues were transferred to a series of tubes containing ice-cold $LaCl_3$/MOPS/buffer (medium d) successively for 1, 1, 2, 2, 4, 10, 20, 30 min, which selectively removes extracellular Ca^{2+} whilst maintaining intracellular Ca^{2+}. Tissues were then blotted on filter paper and weighed, and the radioactivity determined by liquid scintillation counting.

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Media. Media a–c were used at 37 °C, bubbled with 100% O_2; medium d, at ~4 °C, bubbled with 100% O_2. All reagents were analytical grade. (Concentrations in m*M*):

a NaCl, 129.7; KCl, 5.8; $MgCl_26H_2O$, 1.2; $CaCl_2$ $6H_2O$, 1.27; glucose 11.1; MOPS, 10.0 pH 7.4

b NaCl, 49; KCl, 80; $MgCl_26H_2O$, 1.2; $CaCl_26H_2O$, 1.27; glucose 11.1; MOPS 10.0 pH 7.4

c NaCl, 53.1; KCl, 80; $MgCl_26H_2O$, 1.2; glucose 11.1; MOPS 10.0 pH 7.4

d NaCl, 129.7; KCl, 5.8; $MgCl_26H_2O$, 1.2; $LaCl_3$ 10.0; glucose 11.1; MOPS 10.0 pH 7.4

All media were freshly prepared each day from stock solutions. Tamoxifen as free base, (*trans*-1-[4-β-dimethylaminoethoxyphenyl]-1, 2-diphenylbut-1-ene) (ICI plc, Macclesfield, Cheshire) was added from solutions freshly made each day from a 2 mg/ml stock in ethanol kept at <4 °C in the dark.

Results and discussion

Control maximum contractions of the tissue were induced in medium a by changing for 5-min periods to high-K^+ medium b. This produced contractures attributed principally to increased entry of Ca^{2+} ions into the cells [3, 10]. When consistent responses had been obtained the preparations were treated either with 1×10^{-5} *M* or 1×10^{-6} *M* tamoxifen or with vehicle (0.25% ethanol). These treatments were maintained throughout all subsequent procedures. Tamoxifen treatment inhibited the contractions of both myometrium and aorta, so K^+ contractures were repeated at approx 20-min intervals for up to 3 h until equilibrium was attained. At this time the tissues were repeatedly rinsed for 20–30 min in medium c (high K^+ no Ca^{2+}), which completely relaxed the tissues after a brief initial contracture, whilst maintaining depolarisation of the cell membrane. Sequential increments in Ca^{2+} concentration at 5- to 10-min intervals, from threshold for controls at about 4 μ*M* Ca^{2+}, induced successive dose-related increases in contracture up to a maximum at 8 m*M* Ca^{2+}. However, with all the tamoxifen-treated preparations, with the exception of the aorta treated with 10^{-6} *M* tamoxifen, the thresholds were raised and the responses were less than controls at Ca^{2+} concentrations up to 8 m*M*. At higher concentrations of Ca^{2+}, myometrial responses in the presence of 10^{-5} *M* and 10^{-6} *M* tamoxifen were substantially reduced (Fig. 1). Similar inhibition of Ca^{2+}-induced contractions were seen for the aorta, but the contractions to Ca^{2+} in the presence of 10^{-6} *M* tamoxifen changed only marginally. Statistical analysis of the responses obtained after the addition of 1.0 m*M* Ca^{2+}, which is within the physiological range of calcium concentrations and is close to that used for our normal medium a, demonstrated that inhibition by tamoxifen was significant for both tissues at 10^{-5} *M* tamoxifen, but only for the myometrium at 10^{-6} *M* tamoxifen (myometrium, Kruskal-Wallis ANOVA; Mann Whitney U-test vs control: 10^{-5} *M*, $P<0.001$; 10^{-6} *M*, $P<0.02$. Aorta, Kruskal-Wallis ANOVA; Mann Whitney U-test vs control: 10^{-5} *M*, $P<0.001$). This differential effect may reflect the presence of high concentrations of oestrogen receptors in the myometrium [14], which perhaps act to increase the intracellular concentration of tamoxifen. Alternatively, the lower potency of tamoxifen upon the aorta may be a consequence of the relative dependence of the contractile process upon extracellular calcium, as the aorta utilises intracellular stores more readily [4]. Subsequent rinsing in tamoxifen-free medium a restored original responses to high [K^+] levels in the vehicle-treated tissues, but dose-related depression of contractions persisted in the tamoxifen-treated tissues.

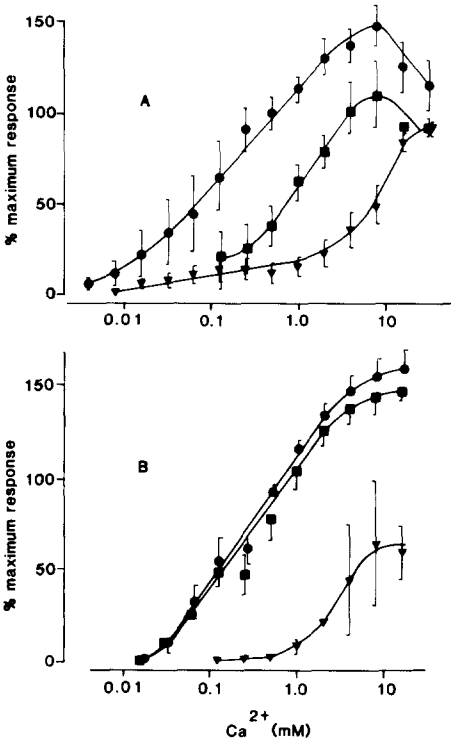

Fig. 1 A, B. Concentration-effect curves to Ca^{2+} of myometrium **(A)** and aorta **(B)** bathed in Ca^{2+}-free depolarising MOPS buffer. The ordinate is the response expressed as a percentage of mean of initial K^+ contractures induced by changing to normal Ca^{2+} depolarising MOPS buffer (medium b) before addition of tamoxifen 10^{-6} *M* (■), or 10^{-5} *M* (▼), or of vehicle (0.25% ethanol; ●). Means ±SEM ($n=4-16$)

Using the same media and similar time sequences, we examined the effect of incubation in tamoxifen upon the uptake of $^{45}Ca^{2+}$ from the media in response to 80 mM K^+ treatment (Table 1). Depolarisation of smooth muscle by 80 m*M* K^+ in both myometrium and aorta significantly increased the uptake of $^{45}Ca^{2+}$, a result consistent with previously published data [10, 22]. Extracellular calcium uptake was responsible for the stimulus-contraction coupling seen in the isolated tissue experiments. The $^{45}Ca^{2+}$ uptake data show that tamoxifen alone has no effect upon Ca^{2+} uptake in the myometrium but may promote uptake in the aorta at 10^{-6} *M*. However, the most important conclusion drawn from this experiment is that tamoxifen could not be demonstrated to significantly inhibit the uptake of extracellular $^{45}Ca^{2+}$ by depolarisation of the cells with K^+. This shows that the influx of Ca^{2+} into the cell is not changed by tamoxifen. This antagonism contrasts with the antagonism produced by drugs such as nifedipine and diltiazem, which act by blocking voltage-dependent calcium channels in the cell membrane [1]. Tamoxifen must therefore antagonise the calcium-mediated contractions demonstrated in Fig. 1 by acting intracellularly.

It is highly likely that the Ca^{2+} antagonism which we have demonstrated is involved in the therapeutic actions of tamoxifen, since calcium is well known to be essentially

Table 1. Effects of tamoxifen on the uptake of $^{45}Ca^{2+}$ into the myometrium and aorta[a]

	Myometrium		Aorta	
	Control	K^+ Stimulated	Control	K^+ Stimulated
Control	0.331 ± 0.028	0.478 ± 0.048	0.117 ± 0.010	0.172 ± 0.006
Tamoxifen 10^{-6} *M*	0.320 ± 0.019	0.454 ± 0.054	0.144 ± 0.015	0.190 ± 0.016
Tamoxifen 10^{-5} *M*	0.293 ± 0.034	0.341 ± 0.020	0.116 ± 0.009	0.154 ± 0.010
Two-way ANOVA: effect of K^+, $F=13.18$; *df* 1,70; $P<0.001$. Effect of tamoxifen N.S. Interaction N.S.			Two-way ANOVA: effect of K^+, $F=25.7$; *df* 1,55; $P<0.001$. Effect of tamoxifen: $F=4.13$; *df* 2,55; $P<0.02$. Interaction N.S.	

[a] Figures displayed indicate mean (± SEM) tissue: medium ratios for $^{45}Ca^{2+}$ in all cases ($n=12-14$ for myometrium; 10 for aorta)

involved with growth and proliferation of neoplasms [8] and mitogens are associated with increased Ca^{2+} influx into the cell [7]. Drugs which block calcium entry into the cell through voltage-dependent channels, and like tamoxifen relax smooth muscle, have also been reported to inhibit tumour cell growth and metastasis [9, 21]. However, their usefulness is probably limited as they are nonselective and have potent hypotensive activity [5]. In addition, the relatively autonomous nature of tumour cells is related to abnormally high intracellular levels of calcium and calmodulin, which lowers the dependence upon extracellular calcium [8]. Consequently, intracellular calcium antagonism may have an important role to play in the treatment of cancer. The present experiments suggest that at high concentrations similar to those that are cytotoxic in vitro, tamoxifen is an intracellular calcium antagonist, a conclusion that is supported by a report that tamoxifen binds to purified calmodulin and inhibits the stimulation of calmodulin-dependent phosphodiesterase [13]. Calmodulin antagonists which also bind to this protein have been shown to inhibit the growth of several tumours, including tumour cells derived from human breast cancer [11, 19, 23, 24].

In view of the association of calcium and neoplastic growth and the antitumour activity of drugs which interfere with cellular calcium metabolism, more attention should be paid to the pharmacological development of compounds to evaluate the therapeutic importance of calcium antagonism for cancer chemotherapy.

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References

1. Bolton TB (1979) Mechanisms of action of transmitters and other substances on smooth muscle. Physiol Rev 59: 606
2. Darbre PD, Curtis S, King RJ (1984) Effects of estradiol and tamoxifen on human breast cancer cells in serum free culture. Cancer Res 44: 2790
3. Edman KAP, Schild HO (1962) The need for calcium in the contractile reponses induced by acetylcholine and potassium in rat uterus. J Physiol (Lond) 161: 424
4. Endo M, Kitazawa T (1985) Separation of 'voltage' and 'receptor' phase for Ca^{2+}. In: Godfraind et al. (eds) Calcium entry blockers and tissue protection. Raven Press, New York, pp 81–89
5. Fleckenstein A (1983) Calcium antagonism in heart and smooth muscle. Wiley, New York
6. Furr BJA, Jordan VC (1984) The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 25: 127
7. Hesketh TR, Moore JP, Morris JDH, Taylor MV, Rogers J, Smith GA, Metcalfe JC (1985) A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. Nature 313: 481
8. Hickie RA, Wei JW, Blyth LM, Wong DY, Klaassen DJ (1983) Cations and calmodulin in normal and neoplastic cell growth regulation. Can J Biochem Cell Biol 61: 934
9. Honn KV, Onoda JM, Pampalona K, Battaglia M, Neagos G, Taylor JD, Diglio CA, Sloane BF (1985) Inhibition by dihydropyridine class calcium channel blockers of tumor cell-platelet-endothelial cell interactions in vitro and metastasis in vivo. Biochem Pharmacol 34: 235
10. Ichida S, Moriyama M, Terao M (1984) Characteristics of Ca influxed through voltage- and receptor-operated channels in uterine smooth muscle. J Pharmacol Exp Ther 228: 439
11. Ito H, Hidaka H (1983) Antitumor effect of a calmodulin antagonist on the growth of solid sacroma 180. Cancer Lett 19: 215
12. Jordan VC (1984) Biochemical pharmacology of antioestrogen action. Pharmacol Rev 36: 276
13. Lam HYP (1984) Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem Biophys Res Commun 118: 27
14. Lippman ME (1983) Antiestrogen therapy of breast cancer. Semin Oncol 10/4 [Suppl 4]: 9
15. Lipton A, Vinijsanun A, Martin L (1984) Acute inhibition of rat myometrial responses to oxytocin by tamoxifen stereo-isomers and oestradiol. J Endocrinol 103: 383
16. Morris ID (1985) Inhibition of intestinal smooth muscle function by tamoxifen and clomiphene. Life Sci 37: 273
17. Reddel RR, Murphy LC, Sutherland RL (1983) Effects of biologically active metabolites of tamoxifen on the proliferation kinetics of MCF-7 human breast cancer cells in vitro. Cancer Res 43: 4618
18. Reddel RR, Murphy LC, Sutherland RL (1984) Factors affecting the sensitivity of T-47D human breast cells to tamoxifen. Cancer Res 44: 2398
19. Sezzi ML, Zupi G, De Luca G, Materazzi M, Bellelli L (1984) Effects of a calcium antagonist (flunarazine) on the in vitro growth of B16 mouse melanoma cells. Anticancer Res 4: 229
20. Taylor CM, Blanchard B, Zava DT (1984) Estrogen receptor-mediated and cytotoxic effects of the antiestrogens tamoxifen an 4-hydroxy tamoxifen. Cancer Res 44: 1409
21. Tsuruo T, Iida H, Nojiri M, Tsukagoshi S, Sakurai Y (1983) Circumvention of vincristine and adriamycin resistance in vit-

ro and in vivo by calcium channel blockers. Cancer Res 43: 2905
22. Van Breeman C, Hwang O, Siegel B (1977) In: Casteels R, Godfraind T, Ruegg JC (eds) Excitation contraction coupling in smooth muscle. Elsevier, Amsterdam, pp 243–252
23. Wei JW, Hickie RA, Klaassen DJ (1983) Inhibition of human breast cancer colony formation by anticalmodulin agents: Trifluoperazine, W7 and W13. Cancer Chemother Pharmacol 11: 86
24. Yoneda T, Kitamura M, Ogawa T, Shin-Ichiro A, Sakuda M (1985) Control of VX_2 carcinoma cell growth in culture by calcium, calmodulin and prostaglandins. Cancer Res 45: 398

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