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ACTION OF ANTIESTROGENS ON THE (Ca^{2+} + Mg^{2+})-ATPase AND Na^+/Ca^{2+} EXCHANGE OF BRAIN CORTEX MEMBRANES

JOÃO O. MALVA, M. CELESTE F. LOPES, M. GRAÇA P. VALE and ARSÉLIO P. CARVALHO

Center for Cell Biology, Department of Zoology and Center for Hormonology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

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Abstract—The effect of tamoxifen (TAM) and other antiestrogens on the Ca^{2+} transport activity of synaptic plasma membranes (SPM) and microsomal membranes isolated from sheep brain cortex was investigated. The maximal (Ca^{2+} + Mg^{2+})-ATPase activity of SPM, which is reached at a pCa of about 6.0–6.5, is decreased by about 30% in the presence of 50 μM TAM, whereas the (Ca^{2+} + Mg^{2+})-ATPase activity of microsomes, which is maximal at a pCa of about 5.0, is decreased by about 90% by 50 μM TAM. In parallel experiments, we observed that the ATP-dependent Ca^{2+} uptake is also affected differently by TAM in the two membrane preparations. We found that 50 μM TAM inhibits SPM Ca^{2+} uptake by about 25–30%, whereas the ATP-dependent Ca^{2+} uptake by the microsomal fraction is inhibited by about 60%. No significant effect of TAM was observed on the Na^+/Ca^{2+} exchange of either membrane system. The results indicate that TAM is a more potent inhibitor of the active, calmodulin-independent Ca^{2+} transport system of the intracellular membranes than of that of the plasma membranes, which is calmodulin-dependent. It appears that TAM inhibits calmodulin-mediated reactions, probably through its binding to calmodulin, as we showed previously. However, the Ca^{2+} transport system of microsomes, which does not depend on calmodulin, is also particularly sensitive to TAM.

In the estrogen target tissues, the biological action of the estrogens is regulated by the hormone level and by the presence of estrogen receptors. Some breast tumors contain high levels of estrogen receptors, and they respond successfully to therapy with antiestrogens [1], which antagonize the estrogens' effect by competing with them at the estrogen receptors [2].

On the other hand, the binding of antiestrogens to estrogen receptors cannot explain all the effects of these drugs [3]. The most well known nonsteroidal antiestrogen, tamoxifen (TAM*), has been shown to alter Ca^{2+}-dependent processes [4–6]. It reduces membrane Ca^{2+} conductances [5] and it binds to calmodulin [6]. Furthermore, it inhibits cAMP phosphodiesterase [7], protein kinase C [8–11], lactate dehydrogenase activity [12], and serotonin and dopamine synaptosomal uptake systems [13]. TAM can also compete with neurotransmitters for their receptors, namely it binds to histamine-like receptors [14], to muscarinic receptors [15] and to dopamine receptors [16]. In addition, TAM also binds to high affinity receptors distinct from estrogen receptors [17–20].

Recently, we showed that TAM binds to calmodulin in a Ca^{2+}-dependent manner and that it inhibits calmodulin stimulation of the erythrocyte membrane Ca^{2+}-ATPase [6]. Therefore, the intracellular Ca^{2+} concentration and its regulation may be involved in the pharmacological effects of this antiestrogen.

In nerve cells, cytoplasmic Ca^{2+} concentration is regulated by Ca^{2+} transport systems localized either in plasma membranes (Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange) or in endoplasmic reticulum [21]. Ca^{2+} is extruded from the cell by the plasma membrane systems, whereas endoplasmic reticulum sequesters the cation from the cytoplasm [21].

In this work, we investigate whether TAM alters the activity of Ca^{2+} transport in both SPM and microsomes isolated from sheep brain. We observed that TAM inhibits calmodulin-dependent (Ca^{2+} + Mg^{2+})-ATPase of synaptic plasma membranes (SPM), but it has a stronger effect on the calmodulin-independent (Ca^{2+} + Mg^{2+})-ATPase of microsomal membranes. It appears that although the TAM effect on some biochemical systems may be calmodulin-mediated, other systems crucial to cell activity, which do not involve calmodulin, are also particularly sensitive to TAM.

MATERIALS AND METHODS

Isolation of synaptic plasma membranes. The SPM fraction was isolated from sheep brain cortex according to the method described by Hajós [22] and by Michaelis and Michaelis [23] and modified by Coutinho *et al.* [24]. The sheep brains were obtained from a local slaughterhouse. The cortex (50 g) was removed and homogenized (1:10) in ice cold buffer (10 mM HEPES–Tris, 0.32 M sucrose,

* Abbreviations: SPM, synaptic plasma membranes; (Ca^{2+} + Mg^{2+})-ATPase, ATP phosphohydrolase (EC 3.6.1.38); EGTA, ethyleneglycol bis(β-*N*,*N*′-tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis-[2-(5-phenyloxazolyl)] benzene; TAM, tamoxifen.

pH 7.4) using a Potter–Elvejhem homogenizer. The homogenate was centrifuged at 1500 *g* for 10 min, and the supernatant (S_1) was collected and centrifuged again at 9000 *g* for 20 min. The supernatant (S_2) was discarded and the pellet was then resuspended in 0.32 M sucrose, layered on the top of 0.8 M sucrose and centrifuged at 9000 *g* for 30 min. The upper phase and the pellet were discarded and the synaptosomal fraction contained in the 0.8 M sucrose phase was diluted with cold demineralized water to 0.4 M sucrose. Then, it was centrifuged at 20,000 *g* for 30 min and the pellet obtained was resuspended in 5 mM HEPES–Tris, pH 8.6. After 15 min of osmotic disruption, we centrifuged the suspension at 8000 *g* for 5 min to remove mitochondria and non-lyzed synaptosomes and the collected supernatant was centrifuged again at 20,000 *g* for 30 min. The resulting SPM pellet was resuspended in sucrose medium containing 10 mM HEPES–Tris (pH 7.4) and 0.32 M sucrose, or in Na^+ medium containing 10 mM HEPES–Tris (pH 7.4), 140 mM NaCl and 1 mM $MgCl_2$. Aliquots of 200 μL containing about 2 mg of protein were frozen in liquid nitrogen and stored at −80°.

Isolation of microsomes. The microsomal fraction was isolated from sheep brain cortex by centrifuging supernatant S_2 at 40,000 *g* for 1 hr. The resulting pellet was resuspended in a medium containing 10 mM HEPES–Tris (pH 7.4), 140 mM NaCl and 1 mM $MgCl_2$ or 10 mM HEPES–Tris (pH 7.4) and 0.32 M sucrose [25], respectively. The protein concentration of SPM and microsomal fractions was determined by the biuret method using bovine serum albumin as standard [26].

Although some contamination may occur in these preparations, we observed that they are characteristically distinct with respect to their (Na^+-K^+)-ATPase and Ca^{2+}-activated ATPase activities, which indicates that they are enriched in different types of biomembranes.

ATPase assay. The ATPase assay was performed by a potentiometric method [27, 28], in which we follow the ATP hydrolysis by recording H^+ production at pH 7.2. All assays were performed at 30° in 2 mL of a medium containing 2 mM Tris, 140 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, variable $CaCl_2$ concentration, 0.5 mM ouabain, 0.125 μg oligomycin and 125 μg of membrane protein at a pH of 7.2. The reaction was started with the addition of 1 mM Mg^{2+}-ATP, and it proceeded during 4 min. The Mg^{2+}-ATPase was determined in the absence of $CaCl_2$ and the total ATPase was measured at several $CaCl_2$ concentrations. The (Ca^{2+} + Mg^{2+})-ATPase was determined by subtracting the Mg^{2+}-ATPase from the total ATPase. Under these experimental conditions, one mole of H^+ is produced by every mole of ATP hydrolyzed. The system was calibrated by adding 100 nmol of HCl–Tritisol.

The results are expressed as nmol ATP hydrolyzed per mg of protein per minute.

ATP-dependent $^{45}Ca^{2+}$ uptake. The Ca^{2+} pump activity was determined by measuring the accumulation of $^{45}Ca^{2+}$ within the vesicles of SPM or of microsomes, respectively [29]. The uptake was measured at 30° using vesicles resuspended in sucrose medium. The uptake solution contained 10 mM HEPES–Tris (pH 7.4), 140 mM KCl, 5 mM $MgCl_2$, 10 μM $^{45}CaCl_2$, oligomycin (1 μg/mg protein) and membrane protein (0.5 mg/mL). The reaction was started by adding 1 mM Mg^{2+}-ATP, and after various time intervals it was stopped by filtering under vacuum, aliquots of 500 μL. Before and after reaction, the filters (Whatman GF/B) were washed twice by filtering 5 mL of a cold solution containing 10 mM Tris–HCl and 0.32 M sucrose, pH 7.4. The Ca^{2+} accumulated was calculated by subtracting the Ca^{2+} bound to the membranes in the absence of Mg^{2+}-ATP from that measured in the presence of Mg^{2+}-ATP. The dried filters were placed in vials with 8 mL of scintillation fluid (7.3 g PPO, 176 mg POPOP and 250 mL Triton X-100 per liter toluene). The radioactivity was counted in a Packard Tri-Carb 2000 CA liquid scintillation spectrophotometer, with dpm correction.

Na^+/Ca^{2+} exchange activity. Na^+/Ca^{2+} exchange activity was measured at 30° using membranes resuspended in Na^+ medium. The uptake medium contained 10 mM HEPES–Tris (pH 7.4), 140 mM KCl, 1 mM $MgCl_2$, 20 μM $^{45}CaCl_2$ and SPM or microsomes (0.5 mg protein/mL) [24]. The uptake was started by adding the membrane vesicles to the uptake medium and, after various time intervals, it was stopped by filtering aliquots of 500 μL through Whatman GF/B filters, which were treated as described above. Accurate values for the activity of Na^+/Ca^{2+} exchange, which are dependent on the Na^+ gradient ($Na^+_{in} > Na^+_{out}$), were obtained by subtracting the Ca^{2+} uptake in Na^+ medium from that in K^+ medium.

The experiments performed in the presence of TAM were controlled by parallel assays in the presence of the solubilizer (ethanol) without the drug.

Reagents. All reagents were of analytical grade. Vanadium-free ATP (disodium salt), oligomycin, ouabain, PPO, POPOP and 17β-estradiol were purchased from the Sigma Chemical Co.; X-537 A was a gift of Dr Julius Berger of Hoffman–La Roche, Nutley, New Jersey, U.S.A.; Nitromifene was from Dr Martin L. Black, Warner–Lambert Co., Michigan, U.S.A.; TAM, OH-TAM and $^{45}CaCl_2$ (11.2 mCi/mg) were obtained from Amersham, trifluoperazine was supplied by Smith, Kline & French, Philadelphia, PA, U.S.A.

RESULTS

Action of antiestrogens on the (Ca^{2+} + Mg^{2+})-ATPase activity of synaptic plasma membranes and microsomal membranes of brain cortex cells

The (Ca^{2+} + Mg^{2+})-ATPase activity of SPM is dependent on Ca^{2+}. It reaches a maximum (≈65 nmol ATP hydrolyzed/mg protein/min) at a pCa value of about 6.0–6.5 and is then inhibited by higher Ca^{2+} concentrations (Fig. 1A). In the presence of TAM (50 μM), the (Ca^{2+} + Mg^{2+})-ATPase activity is maximal (≈55 nmol ATP hydrolyzed/mg protein/min) at higher concentrations of free Ca^{2+} (pCa 5), suggesting that TAM decreases the affinity of the enzyme for Ca^{2+} (Fig. 1A). Indeed, Lineweaver–Burk analysis showed that the $K_{0.5}$(Ca) of the enzyme increased from 233 to 320 nM in the presence of

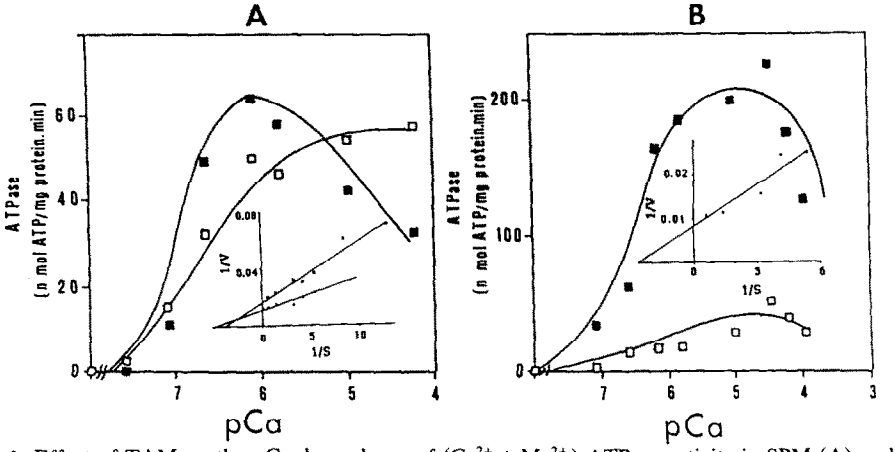

Fig. 1. Effect of TAM on the pCa dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity in SPM (A) and in microsomes (B). The membranes were incubated in the presence or absence of 50 μM TAM in a medium as described in Materials and Methods. The pCa values were adjusted by adding 0.1 mM EGTA and variable amounts of $CaCl_2$. The medium for microsomal ATPase assay also contained Triton X-100 (0.005%). (□) Presence of 50 μM TAM. (■) Absence of TAM. Insert, the Lineweaver–Burk analysis obtained at the Ca^{2+} concentrations which stimulate the ATPase. The results correspond to the mean value calculated from a group of three repetitive assays.

TAM, whereas the V_{max} value is reduced from about 90 to 67 nmol ATP hydrolyzed/mg protein/min (Fig. 1A, insert). Furthermore, TAM appears to protect the enzyme from inhibition by high Ca^{2+} concentrations (Fig. 1A).

In contrast to the mild inhibitory effects of TAM on the $(Ca^{2+} + Mg^{2+})$-ATPase of SPM, TAM greatly reduces (≈90%) the V_{max} value (290 nmol ATP hydrolyzed/mg protein/min) of the microsomal $(Ca^{2+} + Mg^{2+})$-ATPase. The Ca^{2+} affinity ($K_{0.5} \approx$ 500 nM) is not apparently altered, although accurate values could not be determined from the very low ATPase activity observed in the presence of TAM (Fig. 1B). The different behavior of the two membrane ATPases to TAM is compatible with the observations that the microsomal enzyme has biochemical characteristics clearly distinct from those of SPM enzyme. The V_{max} value of the microsomal $(Ca^{2+} + Mg^{2+})$-ATPase is about 210 nmol ATP hydrolyzed/mg protein/min, which was reached at a pCa value (4.5–5) lower than that observed for the SPM enzyme (6–6.5). At higher Ca^{2+} concentrations (pCa 4), the ATPase activity decreases and reaches values of about 125 nmol ATP hydrolyzed/mg protein/min (Fig. 1B). In the presence of TAM (50 μM), the maximum activity of the microsomal ATPase decreases to about 50 nmol ATP hydrolyzed/mg protein/min at pCa 4.5, and no protector effect of TAM is observed against Ca^{2+} inhibition (Fig. 1B).

In order to investigate whether the TAM effect on the ATPase activity of brain membranes is related to a possible regulation of the enzyme by steroids or calmodulin, we studied the $(Ca^{2+} + Mg^{2+})$-ATPase activity of both types of membranes in the presence of estradiol, various antiestrogen drugs and trifluoperazine, respectively (Table 1). In SPM vesicles, 17β-estradiol does not significantly alter the $(Ca^{2+} + Mg^{2+})$-ATPase activity, whereas trifluoperazine (50 μM) inhibits the enzyme activity by about 46%. Similarly to the effect of the calmodulin antagonist trifluoperazine, the antiestrogens, TAM, nitromifen and clomiphene, inhibit the ATPase by about 29%, 65% and 22%, respectively (Table 1). We observed previously that TAM binds to calmodulin [6], but we do not know whether the action of these drugs on the SPM $(Ca^{2+} + Mg^{2+})$-ATPase is calmodulin-mediated.

The antiestrogens have a stronger inhibitory effect on the microsomal $(Ca^{2+} + Mg^{2+})$-ATPase as compared to that on the SPM $(Ca^{2+} + Mg^{2+})$-ATPase (Table 1). We found enzyme inhibition of about 91%, 81% and 55% by TAM, nitromifene and clomiphene, respectively. In contrast to SPM, trifluoperazine (50 μM) has a smaller inhibitory effect (≈25%) than that of the antiestrogens, whereas 17β-estradiol does not inhibit the microsomal $(Ca^{2+} + Mg^{2+})$-ATPase (Table 1). The low sensitivity to trifluoperazine, together with lack of enzyme stimulation by calmodulin [29], indicates that microsomal $(Ca^{2+} + Mg^{2+})$-ATPase of brain cortex cells is not calmodulin-regulated and, therefore, the antiestrogens probably interact directly with the enzyme.

Effect of tamoxifen on the ATP-dependent Ca^{2+} *uptake by synaptic plasma membranes and microsomal membranes of brain cortex cells*

Since TAM inhibits the $(Ca^{2+} + Mg^{2+})$-ATPase activity of brain cortex membranes, we studied whether Ca^{2+} uptake is affected by the drug. Figure 2 shows that the maximal ATP-dependent Ca^{2+} uptake by SPM vesicles is about 4 nmol Ca^{2+}/mg protein/5 min, and that most of this Ca^{2+} is intravesicularly accumulated by the membranes, since it is liberated by the ionophore X-537 A. When TAM (50 μM) is present in the reaction medium, the active Ca^{2+} uptake is inhibited by about 20–30% (Fig. 2A), in agreement with the effect of the drug on the SPM $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 1A).

On the other hand, 50 μM TAM reduces the maximal microsomal Ca^{2+} uptake by about 60%,

Table 1. Action of antiestrogens, 17β-estradiol and trifluoperazine on the $(Ca^{2+} + Mg^{2+})$-ATPase activity of SPM and microsomes*

Ca^{2+}-ATPase (% of maximum activity)	Drug concentration (M)	Tamoxifen	Nitromifen	Clomiphene	17β-Estradiol	Trifluoperazine
SPM	5×10^{-5}	71.6 ± 7.7	35.0 ± 1.1	78.8 ± 11.2	97.0 ± 12.0	54.4 ± 18.8
	10^{-5}	83.9 ± 8.0	88.8 ± 4.2	114.6 ± 20.0	109.0 ± 13.0	80.1 ± 3.5
	10^{-6}	103.5 ± 5.0	95.9 ± 9.4	115.0 ± 21.0	92.5 ± 20.0	106.3 ± 1.8
Microsomes	5×10^{-5}	9.1 ± 2.1	18.7 ± 7.9	45.0 ± 16.0	107.1 ± 13.9	80.9 ± 8.4
	10^{-5}	73.7 ± 11.8	100.6 ± 10	110.7 ± 2.5	115.4 ± 4.7	99.9 ± 13.4
	10^{-6}	102.7 ± 1.0	112.3 ± 0.9	123.4 ± 0.9	114.9 ± 3.0	117.7 ± 5.5

* SPM and microsomal membranes were incubated in a medium as described in Fig. 1, at the optimum pCa values of 6.6 and 4.5, respectively. The results are the means ± SD for four repetitive experiments.

from the value of 1.2 nmol Ca^{2+}/mg protein/5 min to the value of 0.5 nmol Ca^{2+}/mg protein/5 min (Fig. 2B). Although a strong effect is observed, it is not as high as that observed on the ATPase activity (≈90% inhibition), which was measured in the presence of Triton X-100. This difference is probably related to the detergent-induced increase in the ATPase accessibility to TAM. However, due to the leaky effect of Triton X-100 on the membranes, it was not used in the Ca^{2+} uptake experiments.

Effect of tamoxifen on the Na^+/Ca^{2+} exchange activity of synaptic plasma membranes and microsomal membranes of brain cortex cells

We have also studied the effect of TAM in the Na^+/Ca^{2+} exchange activity of both membrane fractions. We observed that, in SPM (Fig. 3A), the maximum Ca^{2+} transport by the Na^+/Ca^{2+} exchanger is about 5 nmol Ca^{2+}/mg protein/10 min and that TAM (50 μM) does not affect Na^+/Ca^{2+} exchange activity. Similarly, no significant effect of 50 μM TAM was detected on the microsomal Na^+/Ca^{2+} exchanger (Fig. 3B) which, in agreement with the previous results of Schellenberg and Swanson [30], has a relatively high Na^+/Ca^{2+} exchange activity (6 nmol Ca^{2+}/mg protein/10 min).

DISCUSSION

The SPM and microsomal $(Ca^{2+} + Mg^{2+})$-ATPases are distinct enzymes that regulate the cytosolic free Ca^{2+} levels in resting neurons [21, 31, 32]. They have been shown to have different characteristics distinguished on the basis of their differential sensitivity to low concentrations of vanadate and to alterations in the Na^+ levels of the reaction medium [29, 33].

The $(Ca^{2+} + Mg^{2+})$-ATPase of SPM is a calmodulin-dependent enzyme [34–36], which, in agreement with previous results [33, 35], was stimulated at low Ca^{2+} levels and inhibited by free Ca^{2+} concentrations higher than 10 μM in the presence of millimolar Mg^{2+} concentrations (Fig. 1A). It has been shown that calmodulin antagonists, such as phenothiazines, inhibit calmodulin-dependent enzymes by interacting with calmodulin in a Ca^{2+}-dependent manner [37–40]. Like phenothiazines, TAM is a potent inhibitor of phosphodiesterase [7] and of the erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase [6]. Indeed, in a previous work, we observed Ca^{2+}-dependent binding of TAM to calmodulin, which correlates well with TAM inhibition of the erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase isolated from red blood cell ghosts [6]. Furthermore, it has been emphasized that structural similarities between TAM and phenothiazines are responsible for the calmodulin antagonism of the antiestrogen [41]. Both types of molecules contain a hydrophobic region and a side chain amino group, which facilitates their interaction with calmodulin [42].

Taking into consideration the calmodulin antagonistic properties of TAM and the calmodulin dependence of the SPM $(Ca^{2+} + Mg^{2+})$-ATPase, it is plausible to assume that the inhibitory effect of TAM on the SPM enzyme is calmodulin-mediated. Indeed, we observed that SPM ATPase inhibition

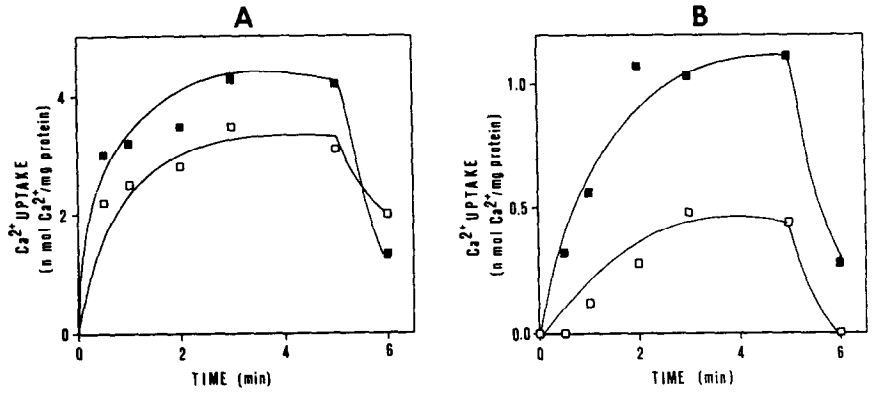

Fig. 2. Effect of TAM on the ATP-dependent Ca^{2+} uptake by SPM (A) and by microsomes (B). The membranes were incubated in the presence or absence of 50 μM TAM in a medium as described in Materials and Methods. At min 5, ionophore X-537 A (5 μM) was added. (■) Absence of TAM. (□) Presence of TAM. The results represent the mean values of three repetitive experiments.

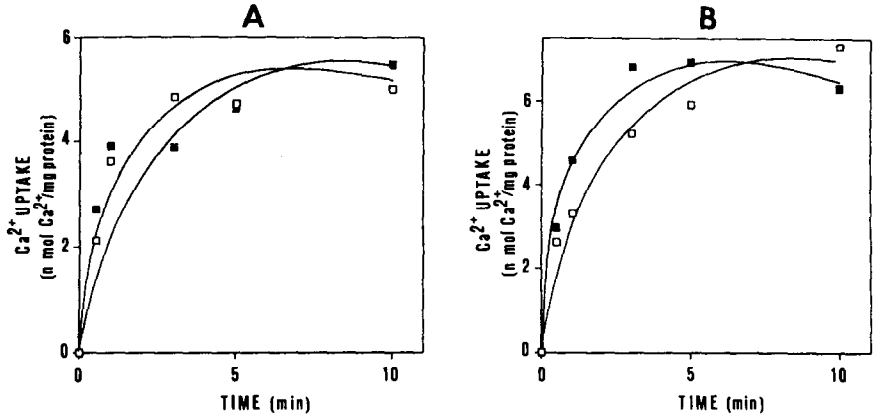

Fig. 3. Action of TAM on the Na^+/Ca^{2+} exchange activity of SPM (A) and microsomes (B). The Na^+/Ca^{2+} exchange activity was measured in Na^+-loaded vesicles by following their $^{45}Ca^{2+}$ uptake in a K^+-containing medium, as described in Materials and Methods. The activity was measured in the absence (■) or in the presence (□) of TAM (50 μM). The results represent the mean values of three repetitive experiments.

by TAM and other antiestrogens is similar to that by trifluoperazine (Table 1), in agreement with previous results obtained with the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase [6].

On the other hand, we observed that TAM decreases the affinity of the SPM ATPase for Ca^{2+} (Fig. 1A), as does trifluoperazine in the heart sarcolemma (Ca^{2+} + Mg^{2+})-ATPase [43]. Although electrostatic effects of hydrophobic amine drugs on the membrane may decrease the Ca^{2+} concentration close to its surface [44], the reduction of the enzyme sensitivity to Ca^{2+} is probably due to calmodulin blockade which prevents the calmodulin-induced transition of the ATPase from a low to a high Ca^{2+} affinity form [43, 45].

We do not know whether calmodulin regulation is an extrinsic or an intrinsic property of the SPM (Ca^{2+} + Mg^{2+})-ATPase, since it has been demonstrated that the plasma membrane Ca^{2+} pump has a domain with an amino acid sequence similar to that of calmodulin [46, 47]. However, our data suggest that the inhibition of the SPM (Ca^{2+} + Mg^{2+})-ATPase by antiestrogens is mediated by a calmodulin-like mechanism. In contrast, the estrogen, estradiol, did not affect the enzyme, probably because its structure does not permit interaction either with the ATPase or with calmodulin.

We have also measured the ATP-dependent Ca^{2+} uptake by SPM vesicles, and we found that TAM has an inhibitory effect on the Ca^{2+} transport activity (Fig. 2), whose magnitude (≈25–30%) is in parallel with that of the TAM effect on the (Ca^{2+} + Mg^{2+})-ATPase.

Regarding the studies with the microsomal (Ca^{2+} + Mg^{2+})-ATPase, we observed that it is not significantly affected by calmodulin antagonists, it is maximally active at 35 μM Ca^{2+}, and it is strongly inhibited by high Ca^{2+} concentrations (Fig. 1B), in agreement with the results obtained by several investigators [29, 48–52].

We observed that TAM has a potent inhibitory effect on the microsomal (Ca^{2+} + Mg^{2+})-ATPase (Fig. 1B and Table 1), which is reflected by a strong reduction of the V_{max} value.

In contrast to the SPM (Ca^{2+} + Mg^{2+})-ATPase, we observed that trifluoperazine is a weaker inhibitor of the microsomal ATPase (Table 1). This is probably related to the absence of stimulation of the enzyme by calmodulin [29], which is in agreement with results obtained in studies with the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase [53]. However, under certain conditions, a small inhibitory effect may occur, which has been attributed to the large partitioning of phenothiazines in the membrane rather than to the involvement of calmodulin in the process [54]. Like trifluoperazine, estradiol had no significant effect on the enzyme activity (Table 1), which indicates that the effects of TAM on the microsomal (Ca^{2+} + Mg^{2+})-ATPase are not mediated either by calmodulin or by steroidal compounds.

On the other hand, we observed that the strong inhibition of the enzyme by TAM is accompanied by a significant inhibition of the Ca^{2+} uptake by microsomal vesicles (Fig. 2), and this probably reflects the fact that the two processes are coupled [55]. These results suggest a specific effect of TAM on the Ca^{2+} pump of the microsomal fraction, which probably represents a direct action of the drug on the enzyme.

In both types of membrane, estradiol has no significant effect on (Ca^{2+} + Mg^{2+})-ATPase activities, so that the TAM effect cannot be explained by disruption of a possible steroidal regulation of the enzymes.

As described above, TAM inhibits both types of Ca^{2+} pump system of the brain membranes, but another Ca^{2+} transport mechanism, Na^+/Ca^{2+} exchange, was completely insensitive to the drug at concentrations below 50 μM (Fig. 3). Na^+/Ca^{2+} exchange is mediated by a carrier which promotes the lowering of Ca^{2+} levels after depolarization [31]. It is activated at higher Ca^{2+} levels than those required to activate (Ca^{2+} + Mg^{2+})-ATPases [56–58] and it is insensitive to several drugs [59], including hydrophobic amine drugs [60, 61] like TAM.

We do not know whether the TAM-induced biochemical effects observed here are involved in the pharmacological action of the drug. Apparently, the TAM concentrations (μM range) required for the effects observed are above the levels (0.3–0.9 μM) of TAM found in the cytosol of breast tumors [62]. On the other hand, cytotoxic effects of TAM (>5 μM) on MCF-7 cells have been demonstrated [63], and side effects have been observed in patients taking TAM [64]. Due to the hydrophobic nature of the antiestrogen drugs, they should be largely partitioned in the membranes, so that it is difficult to determine accurately the concentrations of free drug required to induce the pharmacological effects. However, the results reported here show clearly that some biochemical processes which regulate the intracellular Ca^{2+} concentration are particularly sensitive to TAM through mechanisms involving different target molecules.

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