

# Selective inhibition of oxalate-stimulated $\text{Ca}^{2+}$ transport by cyclopiazonic acid and thapsigargin in smooth muscle microsomes

Peter J. Darby, Chiu-Yin Kwan, and Edwin E. Daniel

**Abstract:**  $^{45}\text{Ca}^{2+}$  uptake and efflux studies were performed on membranes prepared from dog mesenteric artery and rat vas deferens. Oxalate-stimulated, ATP-dependent  $\text{Ca}^{2+}$  uptake in microsomal vesicles, a property characteristic of sarcoplasmic reticulum, was completely inhibited in a concentration-dependent manner by cyclopiazonic acid (0.1–30  $\mu\text{M}$ ) and thapsigargin (10 nM – 10  $\mu\text{M}$ ). Using discontinuous sucrose gradient centrifugation, rat vas deferens microsomes were separated into two fractions, one enriched in plasma membrane (F2), the other enriched in sarcoplasmic reticulum (F3). The F3 fraction had a major increase in  $\text{Ca}^{2+}$  uptake in the presence of oxalate, which was completely inhibited by either cyclopiazonic acid or thapsigargin. In the F2 fraction  $\text{Ca}^{2+}$  uptake in the presence of oxalate was lower than in F3 and was not completely inhibited by thapsigargin and cyclopiazonic acid. Instead, the F2 fraction had a thapsigargin-insensitive and cyclopiazonic acid insensitive, saponin-sensitive component of uptake, which probably represents  $\text{Ca}^{2+}$  uptake by plasma membrane. In the absence of oxalate, the inhibition of  $\text{Ca}^{2+}$  uptake by saponin and cyclopiazonic acid or thapsigargin was additive in the F2 and F3 fractions, suggesting that cyclopiazonic acid and thapsigargin selectively inhibited sarcoplasmic reticulum derived  $\text{Ca}^{2+}$  uptake and did not affect plasma membrane derived  $\text{Ca}^{2+}$  uptake. Measurement of the initial rate of  $\text{Ca}^{2+}$  uptake in the presence and absence of oxalate by rat vas deferens microsomes demonstrated selective inhibition of oxalate-stimulated  $\text{Ca}^{2+}$  uptake by cyclopiazonic acid and thapsigargin.  $\text{Ca}^{2+}$  efflux from rat vas deferens microsomes actively loaded with  $^{45}\text{Ca}^{2+}$  either in the presence or the absence of oxalate was not increased by cyclopiazonic acid or thapsigargin, showing that the inhibition of  $\text{Ca}^{2+}$  accumulation was not due to an increase in  $\text{Ca}^{2+}$  efflux. In both rat vas deferens and dog mesenteric artery, the maximal inhibitory effects of cyclopiazonic acid developed rapidly, whereas for maximal inhibition thapsigargin required pretreatment of microsomes prior to measurement of  $\text{Ca}^{2+}$  uptake. In rat vas deferens microsomes the inhibitory effects of cyclopiazonic acid could be quickly and completely reversed, whereas the effects of thapsigargin were not easily reversed. Collectively, these results suggest selectivity of cyclopiazonic acid and thapsigargin for the sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump. Their selective inhibitory properties and differences in onset and offset of inhibition make cyclopiazonic acid and thapsigargin useful pharmacological tools in the study of the physiological and pathophysiological roles of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump in regulating smooth muscle  $\text{Ca}^{2+}$ .

*Key words:* smooth muscle,  $\text{Ca}^{2+}$  pump, cyclopiazonic acid, thapsigargin, sarcoplasmic reticulum.

**Résumé :** On a examiné l'efflux et la capture de  $^{45}\text{Ca}^{2+}$  sur des membranes préparées d'artère mésentérique de chien et de canal déférent de rat. Dans les vésicules microsomiales, la capture de  $\text{Ca}^{2+}$  fonction de l'ATP, stimulée par l'oxalate, une caractéristique du réticulum sarcoplasmique, a été totalement inhibée, de manière concentration dépendante, par l'acide cyclopiazonique (0,1 – 30  $\mu\text{M}$ ) et la thapsigargine (10 nM – 10  $\mu\text{M}$ ). On a eu recours à la centrifugation sur un gradient discontinu de saccharose pour séparer les microsomes du canal déférent de rat en deux fractions, l'une enrichie en membrane plasmatique (F2), l'autre en réticulum sarcoplasmique (F3). Dans la fraction F3, la capture de  $\text{Ca}^{2+}$  a augmenté significativement en présence d'oxalate, alors qu'elle a été inhibée par l'acide cyclopiazonique ou la thapsigargine. Dans la fraction F2, la capture de  $\text{Ca}^{2+}$  en présence d'oxalate a été plus faible que celle observée dans la fraction F3, et elle n'a pas été totalement inhibée par la thapsigargine ni par l'acide cyclopiazonique. La fraction F2 présentait plutôt une composante de capture sensible à la saponine, insensible à la thapsigargine et l'acide cyclopiazonique, qui illustre probablement la capture de  $\text{Ca}^{2+}$  par la membrane plasmatique. En l'absence d'oxalate, l'inhibition de la capture de  $\text{Ca}^{2+}$  par la saponine et l'acide cyclopiazonique ou la thapsigargine a été additive dans les fractions F2 et F3, ce qui suggère que l'acide cyclopiazonique et la thapsigargine ont inhibé sélectivement la capture de  $\text{Ca}^{2+}$  dérivée du réticulum sarcoplasmique et n'ont pas affecté la capture de  $\text{Ca}^{2+}$  dérivée de la membrane plasmatique. La mesure du taux initial de capture de  $\text{Ca}^{2+}$ , en présence et en l'absence d'oxalate, par les microsomes du canal déférent de rat a démontré une inhibition sélective par l'acide

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cyclopiazonique et la thapsigargine de la capture de  $\text{Ca}^{2+}$  stimulée par l'oxalate. Ni l'acide cyclopiazonique ni la thapsigargine n'ont augmenté l'efflux de  $\text{Ca}^{2+}$  des microsomes du canal déférent de rat chargés de  $^{45}\text{Ca}^{2+}$ , que ce soit en présence ou en l'absence d'oxalate, ce qui indique que l'inhibition de l'accumulation de  $\text{Ca}^{2+}$  n'a pas découlé d'une augmentation de l'efflux de  $\text{Ca}^{2+}$ . Les effets inhibiteurs maximaux de l'acide cyclopiazonique se sont rapidement manifestés dans le canal déférent de rat et l'artère mésentérique de chien, alors que dans le cas la thapsigargine, une inhibition maximale de la capture  $\text{Ca}^{2+}$  n'a pu être obtenue qu'après un prétraitement des microsomes. Dans les microsomes du canal déférent de rat, les effets inhibiteurs de l'acide cyclopiazonique ont pu être renversés rapidement et totalement, alors que ceux de la thapsigargine ne l'ont été que difficilement. Collectivement, ces résultats suggèrent que l'acide cyclopiazonique et la thapsigargine démontrent une sélectivité pour la pompe à  $\text{Ca}^{2+}$  du réticulum sarcoplasmique. En raison de leurs propriétés inhibitrices sélectives et de leurs différences au niveau du déclenchement et de la suppression de l'inhibition, l'acide cyclopiazonique et la thapsigargine constituent des outils pharmacologiques utiles dans l'étude des rôles pathophysiologiques et physiologiques de la pompe à  $\text{Ca}^{2+}$  du réticulum sarcoplasmique dans la régulation du  $\text{Ca}^{2+}$  du muscle lisse.

*Mots clés* : muscle lisse, pompe à  $\text{Ca}^{2+}$ , acide cyclopiazonique, thapsigargine, réticulum sarcoplasmique.  
[Traduit par la Rédaction]

## Introduction

The use of inhibitors believed to be functionally selective for the sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  pump has contributed significantly to our current understanding of the role of the SR in the regulation of cytosolic  $\text{Ca}^{2+}$  levels in smooth muscle (van Breemen 1995; Darby et al. 1993). One such putative SR-selective,  $\text{Ca}^{2+}$  pump inhibitor is cyclopiazonic acid (CPA), a fungal toxin originally shown to accumulate in skeletal muscle (Norred et al. 1985). It was demonstrated that CPA inhibited both  $\text{Ca}^{2+}$  uptake and  $\text{Ca}^{2+}$ -dependent ATPase activity of the skeletal muscle SR (Goeger et al. 1988; Seidler et al. 1989) and had no effect on the kidney and brain  $\text{Na}^+ - \text{K}^+$  ATPase, the gastric  $\text{H}^+ - \text{K}^+$  ATPase, the mitochondrial  $\text{F}_1$  ATPase, the  $\text{Ca}^{2+}$  ATPase of erythrocytes, and the  $\text{Mg}^{2+}$ -activated ATPase of T-tubules and surface membranes of rat skeletal muscle (Seidler et al. 1989).

Another SR-selective  $\text{Ca}^{2+}$  pump inhibitor is thapsigargin (TSG), a naturally occurring sesquiterpene lactone, which inhibits the endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  pump in various nonmuscle cell types, resulting in  $\text{Ca}^{2+}$  release from intracellular stores independent of the production of inositol phosphates or activation of protein kinase C (Thastrup 1990; Kwan et al. 1990). TSG inhibited  $\text{Ca}^{2+}$  uptake and  $\text{Ca}^{2+}$ -dependent ATPase activity in a concentration-dependent manner in rat liver microsomes (Thastrup et al. 1990) and in microsomes isolated from skeletal muscle (Kijima et al. 1991; Lytton et al. 1991; Sagara and Inesi 1991) and cardiac muscle (Kijima et al. 1991; Lytton et al. 1991) with no inhibition of  $\text{Ca}^{2+}$  uptake into plasma membrane (PM) derived vesicles isolated from rat liver or human erythrocytes (Thastrup et al. 1990). Full-length complementary DNA (cDNA) clones encoding all of the SERCA  $\text{Ca}^{2+}$  pumps (SR or ER  $\text{Ca}^{2+}$  ATPase family) were expressed in COS cells and found to be completely inhibited by TSG (Lytton et al. 1991).

Contractility studies using vascular (Deng and Kwan 1991; Low et al. 1991, 1992, 1993; Shima and Blaustein 1992) and airway (Bourreau et al. 1991a) smooth muscles have demonstrated that CPA and TSG inhibit refilling of the agonist- or caffeine-sensitive  $\text{Ca}^{2+}$  store following its depletion in  $\text{Ca}^{2+}$ -free medium. TSG inhibited  $\text{Ca}^{2+}$  accumulation into the inositol trisphosphate ( $\text{IP}_3$ ) sensitive  $\text{Ca}^{2+}$  pools (Bian et al. 1991; Ghosh et al. 1991; Missiaen et al. 1991) and completely inhibited oxalate-stimulated  $\text{Ca}^{2+}$  uptake (Bian et al. 1991) in per-

meabilized cultured smooth muscle cells. Collectively, these results support the hypothesis that CPA and TSG inhibit the SR  $\text{Ca}^{2+}$  pump in smooth muscle.

However, unlike the studies on skeletal muscle, studies of the effects of CPA or TSG on smooth muscle have been limited to functional characterization using intact tissue or isolated cells. Direct evidence of the selective inhibition by these compounds of the smooth muscle SR  $\text{Ca}^{2+}$  pump is not available in the literature despite the common use of CPA and TSG as effective pharmacological tools. The results presented in this paper demonstrate directly, for the first time, that the inhibition of the smooth muscle SR  $\text{Ca}^{2+}$  pump is the most likely mode of action by CPA and TSG in inhibiting ATP-dependent  $^{45}\text{Ca}^{2+}$  uptake into subcellular membrane fractions of rat vas deferens (RVD) and dog mesenteric artery (DMA). RVD was used because it is rich in smooth muscle and its microsomal fractions contain a substantial oxalate-stimulated uptake (Grover and Kwan 1984) and a high density of ryanodine binding sites (Zhang et al. 1993), localized in and characteristic of SR membrane fragments. In addition, the subcellular membrane fractions of RVD have been well characterized by morphological and biochemical criteria (Kwan et al. 1983a). DMA was also studied to demonstrate that the actions of CPA and TSG were equally potent and selective in other smooth muscle types (e.g., vascular smooth muscle).

## Methods

### Tissue handling

Tissue handling and membrane preparation were carried out as previously described for RVD (Kwan et al. 1983a) and for DMA (Kwan et al. 1983b). All procedures were approved by the McMaster University Animal Care Committee. Briefly, male Wistar rats (250–450 g) were killed by cervical dislocation or asphyxiation with carbon dioxide. The whole length of each vas deferens was removed and placed in ice-cold sucrose-imidazole buffer (sucrose, 250 mM; imidazole, 10 mM; pH 7.2). The RVD were cleaned to remove fat, connective tissue, and small blood vessels, then cut open longitudinally, and the epithelial layer was removed with forceps. The smooth muscle was then frozen at  $-20^\circ\text{C}$  until used for preparation of membrane fractions for  $\text{Ca}^{2+}$  uptake experiments.

Mongrel dogs (25–40 kg) of either sex were killed by an overdose of sodium pentobarbital (100  $\text{mg}\cdot\text{kg}^{-1}$ ). The mesenteric vasculature was quickly removed and placed in either ice-cold Krebs solution containing (in mM) NaCl, 119; KCl, 5;  $\text{CaCl}_2$ , 2.5;  $\text{MgCl}_2$ , 2;

NaHCO<sub>3</sub>, 25; NaH<sub>2</sub>PO<sub>4</sub>, 1; and glucose, 11, or ice-cold sucrose-imidazole buffer. The DMA was removed from the mesenteric bed and cleaned on a glass plate kept cold on ice as previously described (Kwan et al. 1983b). The DMA was rinsed with sucrose-imidazole buffer and then frozen at -20°C until used for the preparation of membrane fractions for Ca<sup>2+</sup> uptake experiments.

### Membrane preparation

For the preparation of membranes, the tissues were finely minced with scissors and homogenized using a Polytron PT20 (Brinkmann Instruments Co., Switzerland) at a tissue-volume ratio of 1 g : 15 mL or 1 g : 10 mL for 5 or 10–15 s for RVD and DMA, respectively. The tissue homogenates were fractionated using differential centrifugation. Centrifugation at 900 × g for 10 min removed cell debris, collagen, and nuclei. The supernatant was filtered through four layers of gauze and centrifuged at 9000 × g for 10 min to remove mitochondria. The resulting supernatant was centrifuged at 105 000 × g for 40 min to obtain the crude microsomal pellet (MIC I), which was resuspended in sucrose-imidazole buffer and recentrifuged at 9000 × g for 10 min to further remove fragmented mitochondria. The resulting supernatant, defined as MIC II, was kept on ice until used for Ca<sup>2+</sup> uptake experiments.

For some experiments, the MIC II fraction from RVD was further separated on a sucrose density gradient into fractions enriched in PM or SR. The MIC II fraction was layered on a discontinuous sucrose gradient (15, 30, 40, and 60% sucrose (w/w)) and centrifuged at 105 000 × g for 2 h. The various fractions (F1, F2, and F3) were removed at the sucrose interphases (F1 at the 8 and 15% sucrose interphase, F2 at the 15 and 30% sucrose interphase, and F3 at the 30 and 45% sucrose interphase), diluted to a protein concentration of 0.2–0.5 mg·mL<sup>-1</sup> in sucrose-imidazole buffer (determined by the method of Lowry et al. (1951), using bovine serum albumin as standard), and kept on ice until used for Ca<sup>2+</sup> uptake experiments. Isolated microsomes were used for Ca<sup>2+</sup> uptake experiments within 1–2 h after isolation.

### Enzyme assays

Specific activities of PM and SR marker enzymes were determined on the enriched fractions (F1, F2, and F3, as well as MIC II as control) to determine whether the fractions F2 and F3 were enriched in PM and SR, respectively. For the PM marker enzymes, the specific activities of 5'-nucleotidase (Matlib et al. 1979) and Mg<sup>2+</sup> ATPase (Kwan 1982) were determined spectrophotometrically on the basis of the amount of inorganic phosphate liberated from 5'-AMP over 60 min (for 5'-nucleotidase) or 5'-ATP over 15 min (for Mg<sup>2+</sup> ATPase) at 37°C in a 50 mM imidazole medium buffered at pH 7.2 containing 10 or 5 mM MgCl<sub>2</sub> (for 5'-nucleotidase or Mg<sup>2+</sup> ATPase, respectively). For the SR marker enzyme, the specific activity of NADPH cytochrome *c* reductase was determined by measuring the change in absorbance at 550 nm due to the reduction of cytochrome *c* (Matlib et al. 1979) in a potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM KCN, 50 mM cytochrome *c*, and 0.2 mM NADPH.

### Measurement of calcium uptake

Ca<sup>2+</sup> uptake experiments were conducted at 37°C in a medium containing 250 mM sucrose, 50 mM imidazole, 100 μM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 5 mM sodium azide, 5 mM ATP, 5 mM sodium oxalate (if required), and trace amounts of <sup>45</sup>CaCl<sub>2</sub>. Prior to addition of oxalate and <sup>45</sup>CaCl<sub>2</sub>, the pH was adjusted to 7.2 at 21°C. The Ca<sup>2+</sup> uptake reaction was started by addition of microsomes to the uptake medium, with a final protein concentration of 10–25 μg per reaction tube. Although there was variability in the concentrations between experiments, for those using various fractions, similar protein concentrations were used for the MIC II, F2, and F3 fractions within each experiment. The uptake reaction was stopped after 10 min (except for the experiments represented in Figs. 2 and 4) by rapid filtration through 0.45 μm pore size type HA Millipore filters (Millipore Corp., Bedford,

Mass.) presoaked for 2 h with 0.1 M KCl. The filters were washed twice with 5 mL of ice-cold sucrose-imidazole buffer and removed from the filtration apparatus into individual vials. Scintillation cocktail (Beckman Ready-safe, Beckman Instruments (Canada) Inc., Mississauga, Ont.) was added, and the vials were left overnight in the dark prior to counting of <sup>45</sup>Ca<sup>2+</sup>. Ca<sup>2+</sup> uptake is defined as total Ca<sup>2+</sup> accumulation after subtraction of binding to the microsomes and to the filters. Filter binding was determined by substituting sucrose-imidazole buffer in place of microsomes in the reaction tube. Ca<sup>2+</sup> binding to the microsomes was defined as the <sup>45</sup>Ca<sup>2+</sup> present when the Ca<sup>2+</sup> ionophore A23187 (10 μM) was included in the uptake reaction tube. Filter binding and Ca<sup>2+</sup> binding to the microsomes were determined for each experiment for both control reaction buffer and reaction buffer containing oxalate, done in triplicate. For the experiments using various fractions (MIC II, F1, F2, and F3), Ca<sup>2+</sup> binding to the membranes was determined separately for each fraction. When the effects of saponin were assessed, it was dissolved in ethanol and diluted in water so that 5 μL could be included in the uptake medium at the concentration indicated. The final ethanol concentration was less than 0.2%.

### Measurement of calcium efflux

RVD smooth muscle microsomes were actively loaded (in the presence of ATP) either in the presence or absence of oxalate at 37°C for 30 min, using the uptake medium described above. Efflux was initiated by a 20-fold dilution of the reaction mixture, using efflux medium containing 250 mM sucrose, 10 mM imidazole pH 7.2, and 100 μM EGTA, prewarmed to 37°C. Efflux was stopped by rapid filtration, as was used for the uptake reactions. <sup>45</sup>Ca<sup>2+</sup> in the RVD smooth muscle microsomes was determined as above. Ca<sup>2+</sup> remaining was defined as the total Ca<sup>2+</sup> remaining after subtraction of filter binding. When the effect of CPA (30 μM), TSG (3 μM), or the Ca<sup>2+</sup> ionophore A23187 (10 μM) was tested, each was added to the efflux medium, and the resulting efflux medium containing 30 μM CPA, 3 μM TSG, 10 μM A23187, or no additions (control) was added to the uptake solution to initiate efflux.

### Materials

CPA, TSG, A23187, ATP, and ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) were purchased from the Sigma Chemical Co. (St. Louis, Mo.). CPA, TSG, and A23187 were dissolved in dimethyl sulfoxide (DMSO) and diluted in water so that 5 μL could be added to each reaction tube to achieve the desired final concentration. The final DMSO concentration was less than 0.2%. When added to the efflux buffer, the final DMSO concentration was less than 0.1%. Control experiments showed that these concentrations had no effect on uptake or efflux. <sup>45</sup>CaCl<sub>2</sub> was purchased from NEN Research Products (Boston, Mass.) or ICN Biomedicals Canada Ltd. (St-Laurent, Que.). All other chemicals were of reagent grade and obtained from various commercial sources.

### Statistics

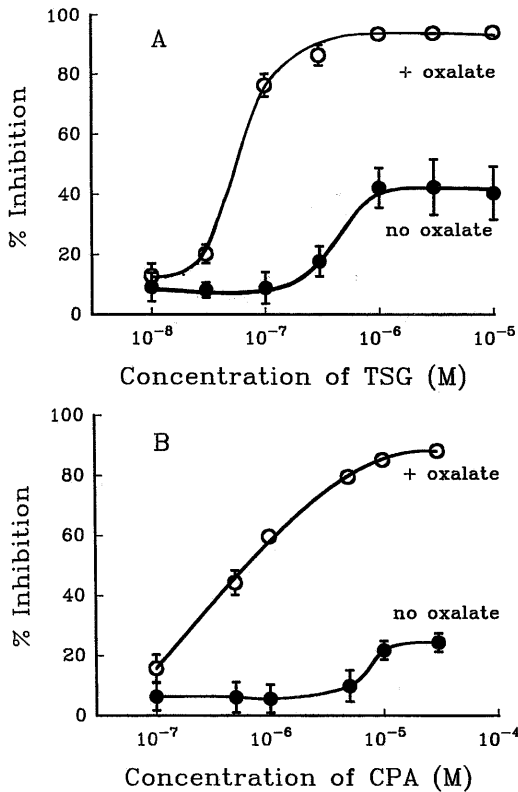
Statistics were calculated using Student's paired *t* tests or repeated-measures ANOVA with Bonferroni post tests as indicated. Differences were considered statistically significant when a *p* value of <0.05 was obtained. Data in figures are means, with bars showing standard error of the means, except when the data point symbol exceeded the size of the error bars.

## Results

### Inhibition of Ca<sup>2+</sup> uptake in the MIC II fraction of RVD by CPA and TSG

In the MIC II fraction from RVD, CPA and TSG inhibited Ca<sup>2+</sup> uptake in the presence or absence of oxalate in a concentration-dependent manner (Fig. 1). In the absence of oxalate,

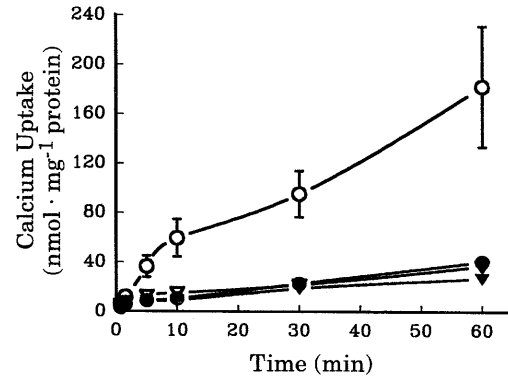
**Fig. 1.** Inhibition of active  $\text{Ca}^{2+}$  uptake by RVD smooth muscle microsomes in the presence (○) and absence (●) of oxalate by varying concentrations of (A) TSG and (B) CPA.  $\text{Ca}^{2+}$  uptake in the presence of oxalate was  $213.3 \pm 48.6$  and  $76.1 \pm 6.6$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in Figs. 1A and 1B, respectively. In the absence of oxalate,  $\text{Ca}^{2+}$  uptake was  $10.7 \pm 2.0$  and  $9.3 \pm 1.0$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in Figs. 1A and 1B, respectively. Each point represents the mean  $\pm$  SEM from 5–8 separate experiments done in triplicate.



the maximal inhibition was  $24.4 \pm 3.1$  and  $42.0 \pm 6.6\%$  by CPA and TSG, respectively.  $\text{Ca}^{2+}$  uptake in the presence of oxalate was maximally inhibited  $88.1 \pm 1.7$  and  $93.2 \pm 1.3\%$  by CPA and TSG, respectively. Oxalate-stimulated  $\text{Ca}^{2+}$  uptake, a unique characteristic of SR-derived microsomes (Brandt et al. 1980; Kwan 1985; Grover 1985), was almost completely inhibited by both compounds ( $97.6 \pm 2.2$  and  $96.6 \pm 1.6\%$  for CPA and TSG, respectively; data not shown). Preliminary data showing concentration-dependent inhibition of oxalate-stimulated  $\text{Ca}^{2+}$  uptake has been previously published elsewhere (Darby et al. 1993). Maximal inhibition was achieved with  $10 \mu\text{M}$  CPA, and the  $\text{IC}_{50}$  was estimated to be  $0.3$  and  $5 \mu\text{M}$  in the presence and absence of oxalate, respectively. Although  $10 \mu\text{M}$  CPA had a maximal inhibitory effect,  $30 \mu\text{M}$  CPA was used for all further studies to ensure complete inhibition. For TSG, maximal inhibition was achieved with a concentration of  $1 \mu\text{M}$ , and the  $\text{IC}_{50}$  was estimated to be  $64$  nM and  $0.4 \mu\text{M}$  in the presence and absence of oxalate, respectively.

The time profiles for  $\text{Ca}^{2+}$  uptake in the MIC II fraction of RVD and its inhibition in the presence of oxalate are shown in Fig. 2. During the entire  $\text{Ca}^{2+}$  uptake period, uptake in the absence of oxalate increased slowly, while uptake in the pres-

**Fig. 2.** Effect of  $30 \mu\text{M}$  CPA on  $\text{Ca}^{2+}$  uptake over a 60-min time period. Uptake in the presence of oxalate (○) continued to increase over the entire 60 min, whereas in the absence of oxalate (●) it levelled off. When  $30 \mu\text{M}$  CPA was included,  $\text{Ca}^{2+}$  uptake in the presence of oxalate (▽) was reduced to levels in the absence of oxalate (▼), indicating complete inhibition of oxalate-stimulated uptake. Each point represents the mean  $\pm$  SEM from 4 separate experiments done in triplicate.



**Table 1.** Distribution of PM and SR marker enzymes in enriched fractions from RVD smooth muscle.

Enzyme	Fraction			
	MIC II	F1	F2	F3
5'-Nucleotidase	76.3±4.6	7.9±0.3	149.3±10.6	54.2±2.9
Mg <sup>2+</sup> ATPase	203.8±11.0	10.8±0.6	418.3±26.2	167.1±10.5
NADPH cytochrome c reductase	0.80±0.13	0.24±0.09	0.89±0.15	1.38±0.20

**Note:** 5'-Nucleotidase and Mg<sup>2+</sup> ATPase activities are expressed as micromoles inorganic phosphate liberated per hour per milligram protein. NADPH cytochrome c reductase activity is expressed as the change in absorbance (at 550 nm) per minute per milligram protein. Each value represents the mean  $\pm$  SEM from 3 separate experiments done in duplicate.

ence of oxalate increased more rapidly. In the presence of  $30 \mu\text{M}$  CPA, the time profiles for  $\text{Ca}^{2+}$  uptake in the presence or absence of oxalate were not distinguishable. These results indicated that CPA inhibited oxalate-stimulated  $\text{Ca}^{2+}$  uptake rapidly, as well as completely.

#### Inhibition of $\text{Ca}^{2+}$ uptake by CPA and TSG in different subcellular membrane fractions

The MIC II fraction of RVD was further separated on a discontinuous sucrose gradient into three fractions (F1, F2, and F3). PM marker enzymes (5'-nucleotidase and Mg<sup>2+</sup> ATPase) showed the highest activity in the F2 fraction, while the SR marker enzyme, NADPH cytochrome c reductase, showed the highest activity in the F3 fraction (Table 1). The levels of enzyme activities and the relative distribution of the enzymes were comparable with previous reports from this laboratory (Kwan et al. 1983a; Bourreau et al. 1991b; Zhang et al. 1993).

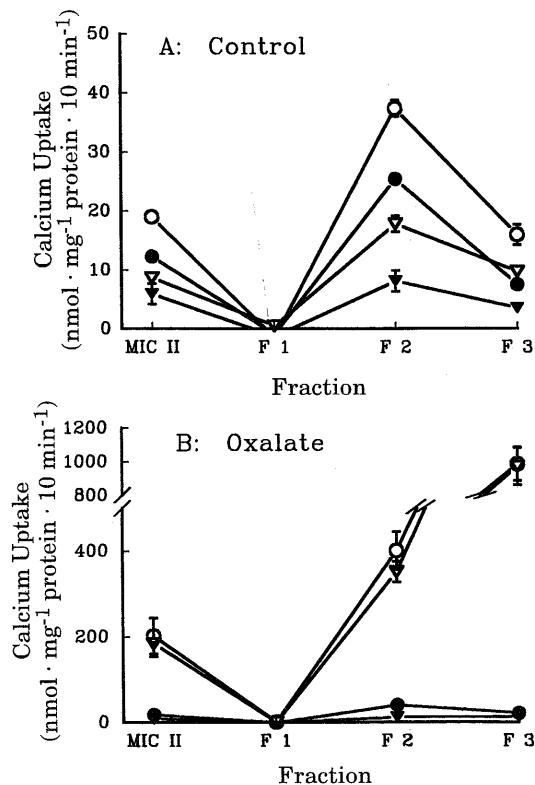
Figure 3A shows that  $\text{Ca}^{2+}$  uptake in the absence of oxalate was the highest in the PM-enriched fraction (F2). Saponin ( $50 \mu\text{g} \cdot \text{mL}^{-1}$ ) inhibited this fraction significantly more than the F3 fraction ( $52.1 \pm 5.3$  and  $37.6 \pm 8.5\%$  for F2 and F3,

**Table 2.** Effect of thapsigargin and saponin on  $\text{Ca}^{2+}$  uptake in the absence and presence of oxalate by enriched fractions from RVD smooth muscle (expressed as % inhibition).

Drugs	Absence of oxalate			Presence of oxalate		
	MIC II	F2	F3	MIC II	F2	F3
TSG, 3 $\mu\text{M}$	32.6 $\pm$ 4.5	31.1 $\pm$ 5.9	61.9 $\pm$ 10.0	91.5 $\pm$ 3.5	88.9 $\pm$ 3.9	97.6 $\pm$ 0.6
Saponin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$	54.6 $\pm$ 23.8	58.1 $\pm$ 9.6	32.8 $\pm$ 2.1	30.1 $\pm$ 15.3	9.4 $\pm$ 5.1	5.8 $\pm$ 2.2
TSG + saponin	84.2 $\pm$ 10.4	89.2 $\pm$ 7.3	90.0 $\pm$ 2.4	98.4 $\pm$ 0.6	97.7 $\pm$ 0.6	99.0 $\pm$ 0.1

**Note:** Data are expressed as percent inhibition of  $\text{Ca}^{2+}$  uptake in each fraction in the absence of any inhibitor. All uptake reactions were stopped after 10 min. Control uptake in the absence of oxalate was 21.6  $\pm$  1.9, 56.8  $\pm$  19.2, and 22.4  $\pm$  7.8 nmol  $\text{Ca}^{2+}\cdot\text{mg}^{-1}$  protein in MIC II, F2, and F3, respectively. TSG showed the greatest inhibition in the F3 fraction ( $p = 0.057$  comparing F2 with F3), while saponin showed the greatest inhibition in the F2 fraction ( $p < 0.05$  comparing F2 with F3). Control uptake in the presence of oxalate was 438.8  $\pm$  245.3, 613.2  $\pm$  252.0, 1273.4  $\pm$  385.6 nmol  $\text{Ca}^{2+}\cdot\text{mg}^{-1}$  protein. TSG inhibited essentially all uptake in the F3 fraction, while in the F2 fraction, there was a residual saponin-sensitive  $\text{Ca}^{2+}$  uptake ( $p < 0.01$  comparing TSG + saponin with TSG alone in the F2 fraction). Saponin by itself had little or no effect on  $\text{Ca}^{2+}$  uptake in the presence of oxalate ( $p > 0.05$  compared with control for both F2 and F3). In the absence of oxalate, the inhibition by TSG alone and saponin alone was additive in both the F2 and F3 fractions ( $p > 0.05$  compared with TSG + saponin for both F2 and F3). Each value represents the mean  $\pm$  SEM from 3 separate experiments done in triplicate.

**Fig. 3.** Effect of 30  $\mu\text{M}$  CPA and 50  $\mu\text{g}\cdot\text{mL}^{-1}$  saponin on  $\text{Ca}^{2+}$  uptake in PM- and SR-enriched fractions in (A) the absence and (B) the presence of oxalate, using the microsomal fraction (MIC II) and 3 fractions (F1, F2, and F3) obtained by discontinuous sucrose gradient centrifugation. The distribution of  $\text{Ca}^{2+}$  uptake in the absence of oxalate is similar to the distribution of PM marker enzymes (Table 1), while the distribution of  $\text{Ca}^{2+}$  uptake in the presence of oxalate is similar to the distribution of the SR marker enzyme (Table 1). Symbols represent  $\text{Ca}^{2+}$  uptake in the absence of all inhibitors ( $\circ$ ), with 30  $\mu\text{M}$  CPA ( $\bullet$ ), with 50  $\mu\text{g}\cdot\text{mL}^{-1}$  saponin ( $\nabla$ ), and with 30  $\mu\text{M}$  CPA plus 50  $\mu\text{g}\cdot\text{mL}^{-1}$  saponin ( $\blacktriangledown$ ). Each point represents the mean  $\pm$  SEM from 3 separate experiments done in triplicate.



respectively), while CPA inhibited the F3 fraction significantly more than the F2 fraction (53.4  $\pm$  3.4 and 32.0  $\pm$  0.8% for F3 and F2, respectively). When both CPA and saponin were included in the  $\text{Ca}^{2+}$  uptake reaction, the inhibition was 78.5  $\pm$  4.8 and 78.7  $\pm$  2.7% for F2 and F3, respectively. These values are not significantly different from those obtained by adding the inhibition by CPA alone to that by saponin alone, suggesting that the effects of CPA and saponin on  $\text{Ca}^{2+}$  uptake are additive in both the F2 and F3 fractions.

Results in Fig. 3B further demonstrate clearly the selectivity of the low concentration of saponin used (50  $\mu\text{g}\cdot\text{mL}^{-1}$ ) for PM-derived  $\text{Ca}^{2+}$  uptake. In the presence of oxalate,  $\text{Ca}^{2+}$  uptake by the various fractions (MIC II and F1, F2, and F3) correlated with the distribution of the SR marker enzyme, NADPH cytochrome *c* reductase (see Table 1), with the highest uptake in the F3 fraction. In the presence of 30  $\mu\text{M}$  CPA, uptake by the F3 fraction was nearly completely inhibited (97.8  $\pm$  0.1%), while in the F2 fraction, there was a saponin-sensitive residual uptake. When saponin was added with CPA to the F2 fraction,  $\text{Ca}^{2+}$  uptake in the presence of oxalate was significantly reduced ( $p < 0.05$  compared with inhibition with CPA alone). Saponin in the absence of CPA had modest inhibitory effects on  $\text{Ca}^{2+}$  uptake in the F2 and F3 fraction when oxalate was present (11.5  $\pm$  3.8 and 3.6  $\pm$  1.4%,  $p > 0.05$  compared with control for both), confirming that saponin was affecting mainly PM-derived  $\text{Ca}^{2+}$  uptake. Collectively, these data are consistent with the existence of a CPA-sensitive, oxalate-stimulated  $\text{Ca}^{2+}$  uptake distinct from a CPA-insensitive, saponin-sensitive  $\text{Ca}^{2+}$  uptake.

The effects of TSG on  $\text{Ca}^{2+}$  uptake by the PM- and SR-enriched fractions were similar to the results seen with CPA (Table 2). In the absence of oxalate, TSG inhibited the F3 fraction to a greater extent than the F2 fraction. In the F2 and F3 fractions, the combination of the inhibition by TSG alone and saponin alone was similar to the level of inhibition by TSG and saponin ( $p > 0.05$ ), suggesting that the effects of TSG and saponin were additive. In the presence of oxalate, TSG almost completely inhibited  $\text{Ca}^{2+}$  uptake in the F3 fraction, while in the F2 fraction, there was a TSG-insensitive, saponin-sensitive component ( $p < 0.01$  compared with TSG alone). These findings suggest that TSG was not inhibiting PM-derived saponin-sensitive  $\text{Ca}^{2+}$  uptake, but was selectively inhibiting  $\text{Ca}^{2+}$  uptake from SR-derived microsomes.

### Inhibition of the initial rate of $\text{Ca}^{2+}$ uptake by CPA and TSG

Using the MIC II fraction from RVD,  $\text{Ca}^{2+}$  uptake was measured in 10-s intervals from 10 to 60 s. Both in the presence (Fig. 4B) and absence (Fig. 4A) of oxalate, initial  $\text{Ca}^{2+}$  uptake proceeded at a constant rate, and was calculated to be  $8.29 \pm 0.72 \text{ nmol } \text{Ca}^{2+} \cdot \text{mg}^{-1} \text{ protein} \cdot \text{min}^{-1}$  in the presence of oxalate and  $3.83 \pm 0.30 \text{ nmol } \text{Ca}^{2+} \cdot \text{mg}^{-1} \text{ protein} \cdot \text{min}^{-1}$  in the absence of oxalate ( $p < 0.005$ ). Both CPA and TSG significantly reduced the rate of  $\text{Ca}^{2+}$  uptake in the presence of oxalate to near that obtained in the absence of oxalate. In the absence of oxalate, the reductions in rates by CPA or TSG were not significant, reflecting the major contribution of  $\text{Ca}^{2+}$  uptake by PM in this fraction. These results confirmed that CPA and TSG inhibited oxalate-stimulated  $\text{Ca}^{2+}$  uptake only, reinforcing the hypothesis that  $\text{Ca}^{2+}$  uptake by the SR-derived microsomes is the site of action of both drugs.

### Differences in the actions of CPA and TSG on $\text{Ca}^{2+}$ uptake

#### Effect of pretreatment

Pretreatment of RVD microsomes at  $21^\circ\text{C}$  with 100 nM TSG for 10 or 60 min significantly increased the inhibition of  $\text{Ca}^{2+}$  uptake in the presence of oxalate. However, the inhibition achieved with  $1 \mu\text{M}$  TSG was similar irrespective of the occurrence or duration of pretreatment (Fig. 5A). On the other hand, the inhibition of  $\text{Ca}^{2+}$  uptake by CPA was not enhanced by pretreatment of the microsomes at  $21^\circ\text{C}$  for 10 or 60 min (Fig. 5B).

#### Reversibility of inhibitory effects of CPA and TSG

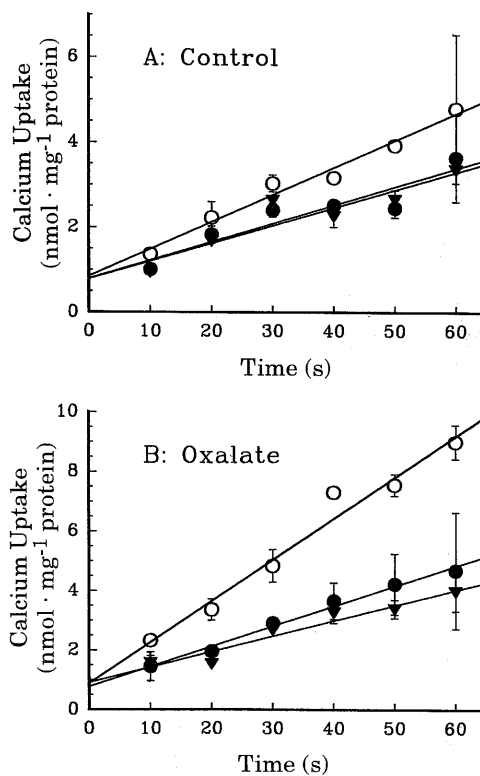
RVD microsomes were pretreated for 10 min with either  $1 \mu\text{M}$  CPA or 100 nM TSG prior to addition of this mixture (i.e., microsomes plus inhibitor) to the  $\text{Ca}^{2+}$  uptake medium for determination of  $\text{Ca}^{2+}$  uptake in the presence of oxalate. For some reaction tubes, no inhibitor was present in the  $\text{Ca}^{2+}$  uptake medium so that dilution of the preincubation medium, which contained the inhibitor, resulted in a 5-fold dilution of CPA or TSG; i.e., the final concentration of CPA or TSG in the  $\text{Ca}^{2+}$  uptake reaction mixture was  $0.2 \mu\text{M}$  or 20 nM, respectively. Some microsomes were pretreated with  $0.2 \mu\text{M}$  CPA or 20 nM TSG, and this mixture was added to the  $\text{Ca}^{2+}$  uptake medium containing similar concentrations of CPA or TSG. In microsomes that were treated throughout with  $1 \mu\text{M}$  CPA,  $\text{Ca}^{2+}$  uptake was inhibited in the presence of oxalate by  $60.7 \pm 3.5\%$  (Fig. 6B). Fivefold dilution of pretreated microsomes (final concentration  $0.2 \mu\text{M}$  CPA) significantly ( $p < 0.005$ ) reduced the level of inhibition of  $\text{Ca}^{2+}$  uptake in the presence of oxalate to the same level as detected in microsomes that were treated with  $0.2 \mu\text{M}$  CPA throughout the experiment ( $24.7 \pm 5.0$  and  $26.2 \pm 5.5\%$ , respectively;  $p > 0.05$ ).

The experiments with TSG gave different results. Fivefold dilution of 100 nM pretreated microsomes, resulting in a final TSG concentration of 20 nM, reduced the level of inhibition of  $\text{Ca}^{2+}$  uptake in the presence of oxalate to  $46.8 \pm 14.2\%$ , compared with  $62.9 \pm 12.8\%$  for microsomes treated throughout with 100 nM TSG. While the level of inhibition of diluted microsomes (final concentration 20 nM TSG) was significantly lower compared with microsomes treated throughout

with 100 nM TSG ( $p < 0.05$ ), it was not reduced to the same level of inhibition as microsomes treated throughout with 20 nM TSG ( $p = 0.076$ ); inhibition was  $11.9 \pm 4.4\%$  in this instance (Fig. 6A).

**Effects of CPA and TSG on  $\text{Ca}^{2+}$  efflux**

We also investigated the effect of CPA and TSG on the efflux of  $\text{Ca}^{2+}$  from RVD microsomal membrane vesicles preloaded with  $^{45}\text{Ca}^{2+}$  in the presence and absence of 5 mM oxalate,

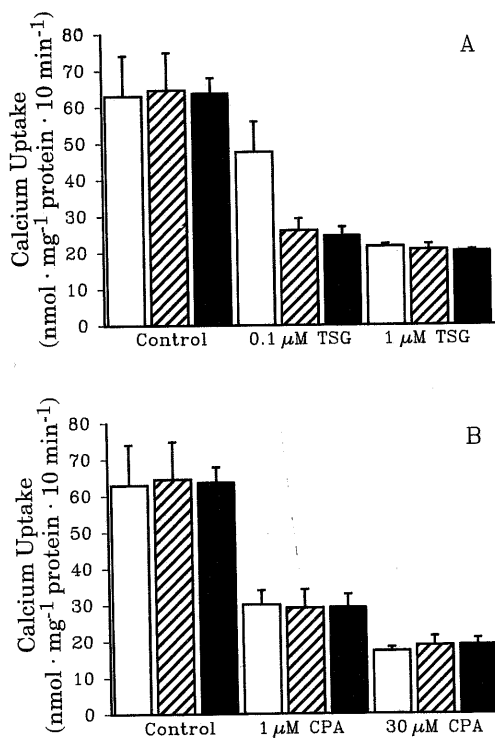


with 100 nM TSG ( $p < 0.05$ ), it was not reduced to the same level of inhibition as microsomes treated throughout with 20 nM TSG ( $p = 0.076$ ); inhibition was  $11.9 \pm 4.4\%$  in this instance (Fig. 6A).

#### Effects of CPA and TSG on $\text{Ca}^{2+}$ efflux

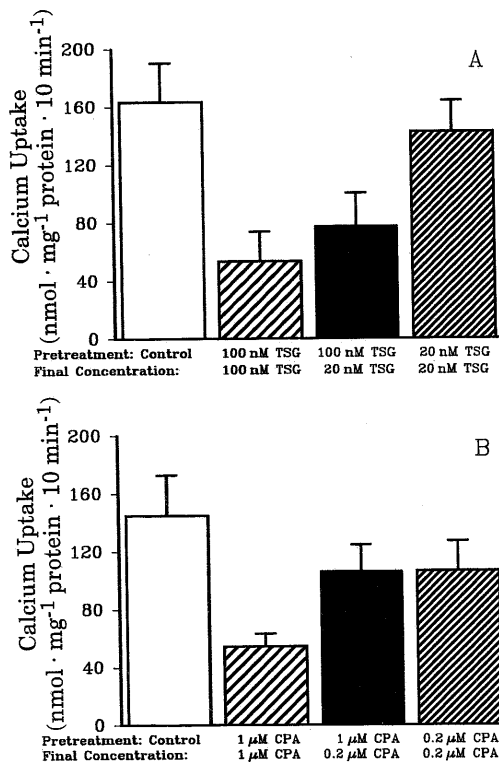
We also investigated the effect of CPA and TSG on the efflux of  $\text{Ca}^{2+}$  from RVD microsomal membrane vesicles preloaded with  $^{45}\text{Ca}^{2+}$  in the presence and absence of 5 mM oxalate,

**Fig. 5.** Effect of pretreatment with (A) thapsigargin or (B) CPA on  $\text{Ca}^{2+}$  uptake in the presence of oxalate in RVD microsomes. The effect of (A) 0.1 and 1  $\mu\text{M}$  TSG and (B) 1 and 30  $\mu\text{M}$  CPA on  $\text{Ca}^{2+}$  uptake in the presence of oxalate was measured to assess whether pretreatment of the microsomes with CPA or TSG was necessary for maximal inhibitory effects. Microsomes were pretreated with CPA or TSG at the concentrations indicated in sucrose-imidazole buffer at 21°C.  $\text{Ca}^{2+}$  uptake (at 37°C) was started by addition of the microsomes to the reaction buffer, which also contained the same concentration of inhibitor as in the pretreatment. The first three bars in both Figs. 5A and 5B (no inhibitor) represent time controls in the absence of any inhibitor, and indicate that time had no effect on  $\text{Ca}^{2+}$  uptake levels. Pretreatment of microsomes with 0.1  $\mu\text{M}$  TSG for 10 min (hatched bars) or 60 min (closed bars) prior to measuring  $\text{Ca}^{2+}$  uptake resulted in a significantly greater inhibition of  $\text{Ca}^{2+}$  uptake compared with microsomes that were not pretreated (open bars,  $p < 0.05$  and  $p = 0.089$  for 10 and 60 min pretreatment, respectively). At 1  $\mu\text{M}$ , TSG did not require pretreatment for maximal inhibition. (B) Pretreatment of microsomes with 1 or 30  $\mu\text{M}$  CPA for 10 min (hatched bars) or 60 min (closed bars) had no effect on the level of inhibition by either concentration. Each bar represents the mean  $\pm$  SEM from 3 or 4 separate experiments done in triplicate.



using the standard  $\text{Ca}^{2+}$  uptake medium, to determine whether changes in membrane permeability to  $\text{Ca}^{2+}$  contributed to the inhibitory effects of CPA and TSG on  $\text{Ca}^{2+}$  uptake. Efflux was initiated by 20-fold dilution with efflux medium or efflux medium containing 30  $\mu\text{M}$  CPA or 3  $\mu\text{M}$  TSG. Maximal efflux of  $\text{Ca}^{2+}$  was determined in the presence of 10  $\mu\text{M}$  A23187 at 10 and 60 min as reference points. Figure 7A shows that in microsomes preloaded in the absence of oxalate,  $\text{Ca}^{2+}$  efflux

**Fig. 6.** Reversibility of the inhibitory effects of (A) TSG and (B) CPA on  $\text{Ca}^{2+}$  uptake in the presence of oxalate in RVD smooth muscle microsomes. Duplicate samples of microsomes were preincubated with 1  $\mu\text{M}$  CPA or 100 nM TSG for 10 min at 21°C prior to addition of this mixture to uptake medium for determination of  $\text{Ca}^{2+}$  uptake (at 37°C). For some tubes (closed bars), the  $\text{Ca}^{2+}$  uptake medium did not contain the inhibitors so that addition of microsomes plus inhibitor resulted in a 5-fold dilution of the inhibitor, i.e., the final concentration of inhibitor was diluted from 1  $\mu\text{M}$  CPA or 100 nM TSG to 0.2  $\mu\text{M}$  CPA or 20 nM TSG. These tubes are referred to as diluted microsomes. In parallel studies, microsomes were pretreated for 10 min with either 0.2  $\mu\text{M}$  CPA or 20 nM TSG prior to addition of this mixture to  $\text{Ca}^{2+}$  uptake medium containing similar concentrations of CPA or TSG (closer hatched bars). The effect of CPA was completely reversible, whereas the effect of TSG was only partially reversible. Two-way ANOVA (Bonferroni post tests) showed no difference between the effects of diluted microsomes (final concentration 0.2  $\mu\text{M}$ ) and those treated with 0.2  $\mu\text{M}$  CPA throughout, while the difference between microsomes treated with 1  $\mu\text{M}$  CPA throughout (wider hatched bars) and diluted microsomes (final concentration 0.2  $\mu\text{M}$ ) was significant ( $p < 0.005$ ). For the experiments with TSG, the level of inhibition was significantly greater ( $p < 0.05$ ) when microsomes were treated with 100 nM TSG throughout compared with those diluted to 20 nM TSG. The level of inhibition for diluted microsomes (final concentration 20 nM TSG) was only marginally greater ( $p = 0.076$ ) than that by microsomes treated throughout with 20 nM TSG. Each column represents the mean  $\pm$  SEM from 5 or 6 separate experiments done in triplicate.



profiles were similar for both the control efflux medium and the efflux medium containing 30  $\mu\text{M}$  CPA or 3  $\mu\text{M}$  TSG.



When 10  $\mu\text{M}$  A23187 was included in the efflux medium, most of the vesicle  $\text{Ca}^{2+}$  was lost by 10 min, and the residual  $\text{Ca}^{2+}$  was probably bound and non-diffusible. Different results were seen with microsomes loaded in the presence of oxalate (Fig. 7B), with both CPA and TSG slightly decreasing the rate of the slow phase (but not the rapid phase) of  $\text{Ca}^{2+}$  efflux. The reduction by 3  $\mu\text{M}$  TSG achieved marginal significance only at the final time point ( $p = 0.083$  at  $t = 60$  min), while the reduction by 30  $\mu\text{M}$  CPA was significant ( $p < 0.05$ ) at both the 30- and 60-min time points. When 10  $\mu\text{M}$  CPA was included in the efflux medium, the results were qualitatively similar to those seen with 3  $\mu\text{M}$  TSG; only the final time point achieved borderline statistical significance ( $p = 0.077$  at  $t = 60$  min, data not shown). These results demonstrate that the inhibition of  $\text{Ca}^{2+}$  accumulation by CPA and TSG (Figs. 1, 2, and 4) was not due to an increase in membrane permeability to  $\text{Ca}^{2+}$  resulting in enhanced  $\text{Ca}^{2+}$  efflux.

### Studies in DMA: effects of CPA and TSG

$\text{Ca}^{2+}$  uptake studies using the MIC II fraction from DMA were qualitatively similar to those obtained with RVD, although it must be noted that the levels of all marker enzyme activities, as well as  $\text{Ca}^{2+}$  uptake, have been shown to be lower for DMA (Kwan et al. 1983b) compared with RVD (Kwan et al. 1983a; Grover and Kwan 1984). The  $\text{Ca}^{2+}$  uptake level in the absence of oxalate was only  $5.4 \pm 0.5$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein, while in the presence of oxalate, the level increased to  $8.4 \pm 1.2$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein. As seen using RVD, both CPA and TSG inhibited  $\text{Ca}^{2+}$  uptake in the presence of oxalate to a greater extent than in the absence of oxalate (Fig. 8). Oxalate-stimulated  $\text{Ca}^{2+}$  uptake was completely inhibited by both compounds (Fig. 8), and maximal inhibition was achieved with 10  $\mu\text{M}$  CPA and 1  $\mu\text{M}$  TSG. Inhibition by CPA was not affected by pretreatment of the microsomes at 21°C prior to the uptake reaction, while for TSG, 10 min pretreatment of the microsomes did increase the level of inhibition of  $\text{Ca}^{2+}$  uptake by lower concentrations of TSG, while it had no effect on the maximal level of inhibition in this vascular tissue (data not shown).

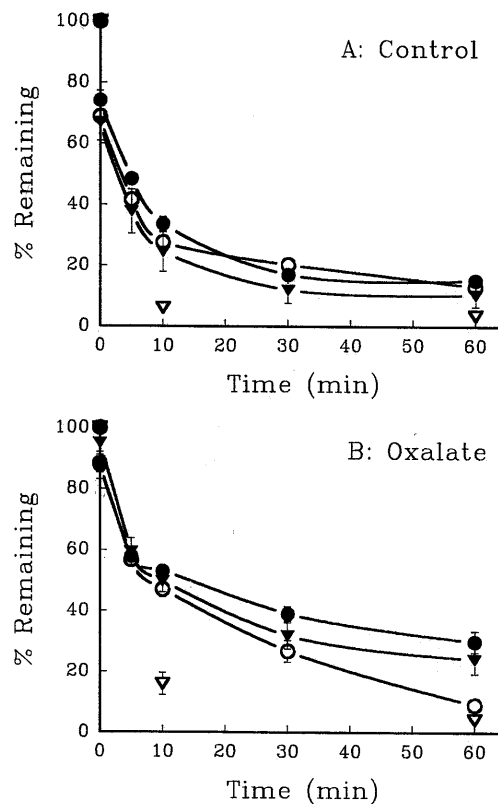
## Discussion

### Actions of CPA and TSG on $\text{Ca}^{2+}$ uptake are selective for SR fragments

The selectivity of CPA and TSG for  $\text{Ca}^{2+}$  pumps of smooth muscle has been inferred either from initial work in skeletal muscle (CPA) and nonmuscle cells (TSG), or from indirect methods of assessing the state of refilling of the intracellular  $\text{Ca}^{2+}$  store. This study on isolated smooth muscle microsomes directly demonstrated the selectivity of CPA and TSG by measuring oxalate-stimulated  $\text{Ca}^{2+}$  uptake, an exclusive property of SR-derived microsomes. This study also demonstrated that saponin-sensitive  $\text{Ca}^{2+}$  uptake, believed to reflect PM-derived uptake, was unaffected by either CPA or TSG.

Using microsomes isolated from RVD smooth muscle, CPA and TSG were shown to inhibit ATP-dependent  $\text{Ca}^{2+}$  accumulation in a concentration-dependent manner both in the presence and absence of oxalate and to completely inhibit oxalate-stimulated  $\text{Ca}^{2+}$  uptake. Maximal inhibition was achieved with 10–30  $\mu\text{M}$  CPA and 1–3  $\mu\text{M}$  TSG. These re-

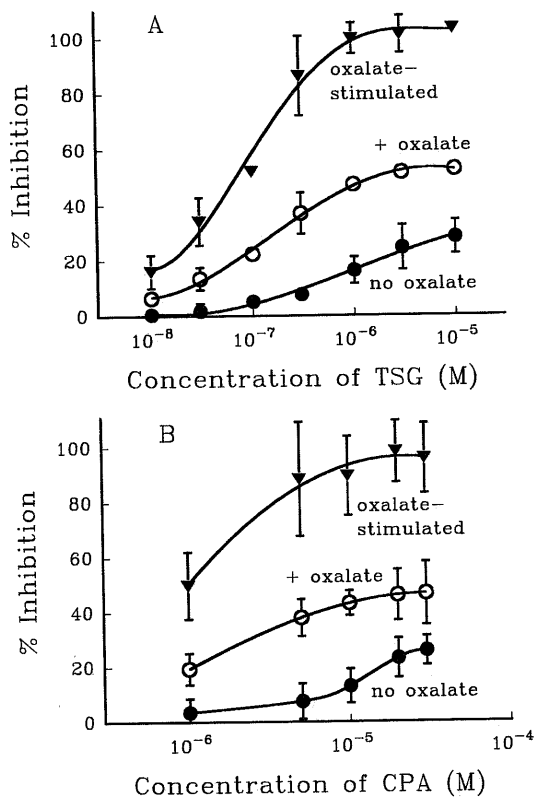
Fig. 7. Effect of 30  $\mu\text{M}$  CPA and 3  $\mu\text{M}$  TSG on  $\text{Ca}^{2+}$  efflux from RVD microsomes actively loaded in (A) the absence and (B) the presence of oxalate. (A) Both 30  $\mu\text{M}$  CPA (●) and 3  $\mu\text{M}$  TSG (▼) had no effect on efflux from microsomes loaded in the absence of oxalate compared with controls (○). Maximal efflux of  $\text{Ca}^{2+}$  was determined by including 10  $\mu\text{M}$  A23187 in the efflux medium (▽). Efflux from microsomes loaded in the presence of oxalate (Fig. 7B) was almost complete by 60 min (○) but was slowed down when 3  $\mu\text{M}$  TSG was added to the efflux medium (▼), with only the final time point (60 min) showing borderline statistical significance ( $p = 0.058$  using Student's paired  $t$  test). When 30  $\mu\text{M}$  CPA was added to the efflux medium (●), efflux was slowed down to a greater extent than seen with TSG ( $p < 0.05$  at 30 and 60 min compared with controls, using Student's paired  $t$  test). When 10  $\mu\text{M}$  CPA was included in the efflux medium, the results were qualitatively similar to those seen with 3  $\mu\text{M}$  TSG (data not shown). Initial  $\text{Ca}^{2+}$  levels (0 min) of microsomes loaded in the absence of oxalate were  $26.1 \pm 3.1$ ,  $29.5 \pm 4.7$ , and  $22.7 \pm 4.0$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in control, 30  $\mu\text{M}$  CPA, and 3  $\mu\text{M}$  TSG, respectively. Initial  $\text{Ca}^{2+}$  levels (0 min) of microsomes loaded in the presence of oxalate were  $153.2 \pm 35.0$ ,  $144.2 \pm 39.1$ , and  $162.2 \pm 67.2$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in control, 30  $\mu\text{M}$  CPA, and 3  $\mu\text{M}$  TSG, respectively. Each point represents the mean  $\pm$  SEM from 3 separate experiments done in triplicate.



sults are consistent with concentrations required to inhibit SR  $\text{Ca}^{2+}$  uptake in functional studies (Deng and Kwan 1991; Bourreau et al. 1991a, 1993; Low et al. 1991, 1992, 1993; Shima and Blaustein 1992; Uyama et al. 1992; Bian et al. 1991; Ghosh et al. 1991; Missiaen et al. 1991).



**Fig. 8.** Inhibition of  $\text{Ca}^{2+}$  uptake in the presence (○) and absence (●) of oxalate and oxalate-stimulated  $\text{Ca}^{2+}$  uptake (▼) by (A) TSG and (B) CPA, using DMA microsomes.  $\text{Ca}^{2+}$  uptake in the presence of oxalate was  $8.0 \pm 0.9$  and  $8.4 \pm 1.2$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in Figs. 8A and 8B, respectively. In the absence of oxalate,  $\text{Ca}^{2+}$  uptake was  $5.2 \pm 0.5$  and  $5.4 \pm 0.5$  nmol  $\text{Ca}^{2+} \cdot \text{mg}^{-1}$  protein in Figs. 8A and 8B, respectively. Each point represents the mean  $\pm$  SEM from 3 (Fig. 8A) or 4 (Fig. 8B) separate experiments done in triplicate.



The mechanism of action of CPA and TSG was not limited to RVD smooth muscle. In DMA microsomes, although much lower SR  $\text{Ca}^{2+}$  uptake was found, CPA and TSG completely inhibited oxalate-stimulated  $\text{Ca}^{2+}$  uptake (Fig. 8). The estimated  $\text{IC}_{50}$  values for the compounds were similar between these two tissues, suggesting that the lower levels of inhibition were not a result of a shift in sensitivity of the DMA microsomes to these inhibitors but were due to the inherent tissue differences in the SR content and (or) the  $\text{Ca}^{2+}$  pump activities. Both CPA and TSG inhibited  $\text{Ca}^{2+}$  uptake in a concentration-dependent manner, and at maximum effect, inhibition of oxalate-stimulated  $\text{Ca}^{2+}$  uptake in DMA was complete.

$\text{Ca}^{2+}$  uptake by PM vesicles isolated from erythrocytes (Sarkadi 1980) and skeletal muscle sarcolemma (McNamara et al. 1971), as well as preparations of skeletal muscle T-tubules (Brandt et al. 1980), is reported to be unaffected by oxalate, and similar results are found in smooth muscle PM vesicles (Grover 1985). The selective inhibition of oxalate-stimulated  $\text{Ca}^{2+}$  uptake by CPA and TSG is therefore presumed to be due to inhibition of the SR but not the PM  $\text{Ca}^{2+}$  pump.

To further evaluate the hypothesis that CPA and TSG preferentially inhibit the SR  $\text{Ca}^{2+}$  pump, the crude microsomal

fraction from RVD was further separated into two subfractions, F2 and F3, enriched in PM and SR membrane vesicles, respectively. As predicted, on the basis of the membrane compositions of the two fractions, CPA and TSG inhibited  $\text{Ca}^{2+}$  uptake by the SR-enriched fraction more completely, while saponin inhibited that by the PM-enriched fraction more completely. Similar to previous studies (Kwan 1985), the findings of the present study (see Fig. 3B and Table 2) demonstrate that saponin ( $50 \mu\text{g} \cdot \text{mL}^{-1}$ ) did not affect the oxalate-stimulated  $\text{Ca}^{2+}$  uptake but preferentially inhibited PM-derived  $\text{Ca}^{2+}$  uptake. In the presence of oxalate, CPA and TSG completely inhibited  $\text{Ca}^{2+}$  uptake by the SR-enriched fraction, but in the PM-enriched fraction, there was a significant CPA- and TSG-insensitive, saponin-sensitive component thought to represent  $\text{Ca}^{2+}$  uptake by PM-derived microsomes. In addition, in the absence of oxalate, the effect of saponin and CPA or TSG was additive in either the PM- or SR-enriched fractions (see Fig. 3A and Table 2). When both saponin and CPA or TSG were included in the  $\text{Ca}^{2+}$  uptake reaction in the absence of oxalate, the level of inhibition was not quite 100% as expected, since saponin at  $50 \mu\text{g} \cdot \text{mL}^{-1}$  will not completely inhibit PM-derived  $\text{Ca}^{2+}$  uptake (Kwan 1985). For complete inhibition, more than  $100 \mu\text{g} \cdot \text{mL}^{-1}$  is required, but these concentrations also significantly inhibit SR-mediated  $\text{Ca}^{2+}$  uptake. However, the additivity of saponin and CPA or TSG suggests that a CPA- or TSG-insensitive, PM-derived, saponin-sensitive  $\text{Ca}^{2+}$  uptake was present in these membranes in addition to the SR-mediated, oxalate-stimulated  $\text{Ca}^{2+}$  uptake.

#### Site of action of CPA and TSG on SR is the $\text{Ca}^{2+}$ pump

Although an increased  $\text{Ca}^{2+}$  efflux might account for the inhibition of  $\text{Ca}^{2+}$  accumulation by CPA or TSG (Fig. 7), this was not the case. Both CPA and TSG slightly decreased the slow phase of  $\text{Ca}^{2+}$  efflux. Inhibition of  $\text{Ca}^{2+}$  efflux by CPA has been shown previously by Goeger and Riley (1989) in skeletal muscle SR vesicles, and by Missiaen et al. (1992) in saponin-skinned cultured smooth muscle cells. Missiaen et al. (1992) suggested from their study that CPA caused a nonselective effect on the SR membrane. However, CPA has no effect on  $\text{D-}^{14}\text{C}(\text{U})$ sorbitol efflux in skeletal muscle SR vesicles (Goeger and Riley 1989), suggesting that CPA does not inhibit SR membrane permeability nonselectively. The initial rate (Fig. 4) and time course (Fig. 2) of  $\text{Ca}^{2+}$  uptake by RVD microsomes were reduced in the presence of oxalate by CPA and TSG to levels seen in the absence of oxalate. These findings strongly suggest that the site of action of CPA and TSG is primarily on  $\text{Ca}^{2+}$  uptake by the pump. The decrease in  $\text{Ca}^{2+}$  efflux may also be due to an action on the pump; i.e., inhibition of efflux through the SR  $\text{Ca}^{2+}$  pump. A  $\text{Ca}^{2+}$  conducting channel has been reported in blebs of skeletal muscle SR (Wang et al. 1992), which was blocked only by several SR  $\text{Ca}^{2+}$  pump inhibitors (vanadate,  $\text{AlF}_4^-$ , and CPA). Thus this  $\text{Ca}^{2+}$  conducting channel may be a form of the SR  $\text{Ca}^{2+}$  pump. In skeletal muscle, a transmembrane channel within the SR  $\text{Ca}^{2+}$  pump has been proposed by de Meis and Inesi (1992). Passive  $\text{Ca}^{2+}$  efflux, uncoupled to the synthesis of ATP, occurred through this channel and could be blocked by TSG, demonstrating that this channel was a part of the SR  $\text{Ca}^{2+}$  pump. Our experimental conditions for  $\text{Ca}^{2+}$  efflux measurements have very low levels of  $\text{Mg}^{2+}$ , ADP, and  $\text{P}_i$ , and may allow uncoupled  $\text{Ca}^{2+}$  efflux through the SR  $\text{Ca}^{2+}$  pump (de Meis et al. 1990), which is

sensitive to CPA. However, further studies are needed to evaluate this hypothesis.

We have not been able to demonstrate the effects of TSG or CPA on  $\text{Ca}^{2+}$  pump ATPase activities in these smooth muscle membranes because studies of the  $\text{Mg}^{2+}$ -stimulated  $\text{Ca}^{2+}$  ATPase are very difficult with smooth muscle SR preparations. These have low proportions of SR membrane and high activities of other  $\text{Mg}^{2+}$  ATPases, which swamp  $\text{Mg}^{2+}$ -stimulated,  $\text{Ca}^{2+}$  ATPases (Kwan 1982).

### Differences between the actions of CPA and TSG

To obtain its maximal inhibitory effect, pretreatment of the microsomes with TSG but not CPA was required prior to initiating the  $\text{Ca}^{2+}$  uptake reaction (Fig. 5). The full action of TSG required 1–2 h of equilibration in intact vascular tissues but only minutes in isolated smooth muscle cells (Low et al. 1991). Our results demonstrate that maximal inhibition of microsomal  $\text{Ca}^{2+}$  uptake required less than 10 min but more than a few seconds, similar to that in isolated cells (Kwan et al. 1990). Whole tissues are thicker and have connective tissue, which slows the rate of diffusion and increases the length of the diffusion path for TSG to reach and enter cells. The full effects of CPA occurred rapidly in contractility studies (Deng and Kwan 1991; Bourreau et al. 1991a, 1993; Low et al. 1992), and in agreement with these results, the  $\text{Ca}^{2+}$  uptake studies shown here also demonstrate the rapid onset of action of CPA.

The requirement for pretreatment with TSG for maximal inhibition of  $\text{Ca}^{2+}$  uptake in RVD microsomes is in contrast to results using skeletal and cardiac microsomes and microsomes isolated from COS cells transfected with full-length cDNA clones of each of the SERCA  $\text{Ca}^{2+}$  pumps (Lytton et al. 1991). While our studies indicated that pretreatment enhanced maximal inhibition by TSG in RVD microsomes, Lytton et al. (1991) demonstrated that the onset of inhibition by TSG was rapid (within seconds) and that the occurrence of maximal effect did not require pretreatment with the microsomes prior to determination of  $\text{Ca}^{2+}$  uptake. The explanation of the differences in the onset of TSG action between membranes of different cell types is unclear.

The offset, like the onset, of effects of TSG in contractility studies was slow (Low et al. 1991, 1993). In contrast, the effects of CPA, which have a rapid onset, were readily reversible (Deng and Kwan 1991; Bourreau et al. 1991a, 1993; Low et al. 1992). The differences in offset of CPA and TSG observed in these functional studies were also demonstrated at the subcellular level. The inhibition by CPA was completely and rapidly reversed, suggesting rapid dissociation of CPA from the SR  $\text{Ca}^{2+}$  pump. In contrast, the inhibition of  $\text{Ca}^{2+}$  uptake by TSG was only partially or slowly reversible. These results testing pretreatment and reversibility in subcellular membranes suggest that the rapid onset and reversibility of CPA effects and the slow onset and poor reversibility of TSG effects in contractility experiments are, in part, properties of the differential interaction of these two agents with the SR membrane and  $\text{Ca}^{2+}$  pump.

### Conclusions

Our data are consistent with the conclusion that CPA and TSG act selectively to inhibit  $\text{Ca}^{2+}$  uptake by SR in smooth muscle

as in striated muscles and support the hypothesis that CPA and TSG selectively inhibit the SR but not the PM  $\text{Ca}^{2+}$  pump in smooth muscle. However, our results revealed differences in the onset and offset of action of CPA and TSG, which must be taken into account when they are used as experimental tools.

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