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# α-Adrenergic Blockade and Inhibition of A23187 Mediated $Ca^{2+}$ Uptake by the Calcium Antagonist Verapamil in Rat Liver Cells

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SUMMARY

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The effects of verapamil on basal and hormone-induced glycogenolysis, phosphorylase activation, $^{45}Ca$ efflux and calcium content in isolated rat liver parenchymal cells were studied. The agent inhibited the stimulatory effects of epinephrine and phenylephrine on all these parameters, but did not modify the actions of vasopressin, glucagon, exogenously-added cAMP and low concentrations of the ionophore A23187. It inhibited calcium uptake due to high concentrations ($>10^{-6}$ M) of A23187, but did not modify the activation of phosphorylase. Verapamil inhibited the binding of $4 \times 10^{-8}$ M [$^3$H]epinephrine to liver plasma membranes in the presence of the β-adrenergic blocker, propranolol. The concentration for half-maximal inhibition of binding ($2 \times 10^{-5}$ M) was very similar to that producing half-maximal inhibition of the effects of $4 \times 10^{-8}$ M epinephrine on phosphorylase activation or calcium efflux in hepatocytes. Verapamil was without significant effect on the reaccumulation of calcium by cells previously depleted of calcium by treatment with EGTA. It is concluded that verapamil acts as an α-adrenergic antagonist in rat liver and has no detectable effects on transmembrane $Ca^{2+}$ flux.

## INTRODUCTION

Much evidence is accumulating which demonstrates that $Ca^{2+}$ ions play an important role in the α-adrenergic activation of glycogenolysis in rat liver (1–4) and that stimulation of α-receptors increases transmembrane flux of $Ca^{2+}$ ions (1, 2, 4). The calcium antagonist verapamil (α-isopropyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxyphenyl acetonitrile hydrochloride) and its methoxy derivative D600 have been used to study $Ca^{2+}$ ion translocation across plasma membranes of cardiac and smooth muscle (5–7). However, there has only been one brief report dealing with the effects of these agents in liver and this dealt with gluconeogenesis (8). This communication presents the results of a study on the effects of verapamil on phosphorylase activation and $Ca^{2+}$ flux induced by α-adrenergic agonists and the divalent cation ionophore A23187 in isolated rat liver parenchymal cells.

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## MATERIALS AND METHODS

Isolated liver parenchymal cells were prepared from fed rats as previously described



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(9). Cells were washed and incubated in Krebs-Henseleit bicarbonate buffer containing 1.5% (w/v) gelatin and 2.65 mM $Ca^{2+}$ (as measured by atomic absorption spectroscopy). Total cell calcium, $^{45}Ca$ efflux exchange, glucose output and phosphorylase *a* levels were measured as previously described (2, 4, 9).

Incubations with cells were performed in triplicate with duplicate samples being taken for assay. Each experiment was repeated from three to five times with different cell preparations.

Binding of [$^3$H]epinephrine to rat liver plasma membranes prepared by the method of Song *et al.* (10) was measured as follows. Membranes were suspended in 50 mM Tris-HCl buffer (pH 7.5) to give approximately 2 mg of protein per ml. The incubation contained 150 μl of membrane suspension, 0.8 mM ascorbate, 3 mM catechol, 10 mM $MgCl_2$, 10 μM (±) propranolol, 40 nM (±) [$^3$H]epinephrine, ± 10 μM phentolamine in a volume of 200 μl. Incubation was for 30 min at 25° in 12 mm × 75 mm polypropylene tubes. Aliquots (90 μl) were withdrawn at the end of incubation and layered on 1 ml of Tris-HCl buffer (pH 7.5) containing 1% (w/v) bovine serum albumin (Pentex) in 1.5 ml polypropylene microcentrifuge tubes (Eppendorf) and rapidly centrifuged at 13,000 × *g* for 5 min at 2–4° in a Beckman microcentrifuge. The supernatant was withdrawn and the pellet carefully washed with 1 ml of 10% (w/v) sucrose. The lower end of the tube was cut off into 10 ml of ACS scintillation fluid (Amersham/Searle) and counted.

Specific α-adrenergic binding is defined as the difference between the amount of [$^3$H]ligand bound in the presence and absence of 10 μM phentolamine. It generally represented 60% of total binding. More complete details and validation of the assay are described elsewhere (11, 12).

(±)-[7-$^3$H(N)]-Epinephrine tartrate was from New England Nuclear. Verapamil was a generous gift from Knoll Pharmaceutical Co., Whippany, N.J. Sotalol hydrochloride was from Regis Chemical Co. Sources of other materials and reagents are described elsewhere (2, 4, 9).

## RESULTS

*Effects of verapamil on hormone-stimulated glycogenolysis in hepatocytes.* Figure 1 shows the effects of verapamil ($10^{-4}$ M) on glucose release from hepatocytes incubted with phenylephrine, glucagon, exogenous cAMP,[1] the divalent cation ionophore A23187, epinephrine (plus propranolol) and vasopressin. Verapamil markedly inhibited the stimulation of glycogenolysis induced by phenylephrine and epinephrine, the two agents exhibiting α-adrenergic activity, but had minimal or no effects on the action of the other agents. No effects were seen on $10^{-5}$ M phenylephrine with $10^{-5}$ M verapamil. At $2 \times 10^{-4}$ M and $4 \times 10^{-4}$ M the agent caused similar changes to those in Fig. 1, except that basal glycogenolysis was increased (data not shown).

To gain evidence for or against the idea that verapamil inhibited the actions of phenylephrine and epinephrine by blocking $Ca^{2+}$ influx, the effects of increasing the extracellular $Ca^{2+}$ concentration were examined, as in other studies (13, 14). Table 1 shows that increasing the $Ca^{2+}$ concentration of the medium from 2.65 mM to 5 or 10 mM did not reverse verapamil inhibition of phenylephrine-stimulated glycogenolysis. This suggests either that verapamil does not inhibit α-adrenergic activation of glycogenolysis by reducing $Ca^{2+}$ influx or that $Ca^{2+}$ influx is not involved in this action of α-agonists.

*Effects of verapamil on epinephrine binding to liver plasma membranes.* In an effort to explain the potent inhibition by verapamil of glycogenolysis stimulated by α-agonists, but not by other agents, the possibility was entertained that the calcium antagonist was also an α-adrenergic blocker. To test this, its ability to inhibit $4 \times 10^{-8}$ M [$^3$H]epinephrine binding to liver plasma membranes in the presence of $10^{-5}$ M propranolol, $8 \times 10^{-4}$ M ascorbate and $3 \times 10^{-3}$ M catechol was examined. As described elsewhere in detail (11, 12), this measures binding to sites with the charac-

[1] The abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis (β-aminoethylether)N,N′-tetraacetic acid.

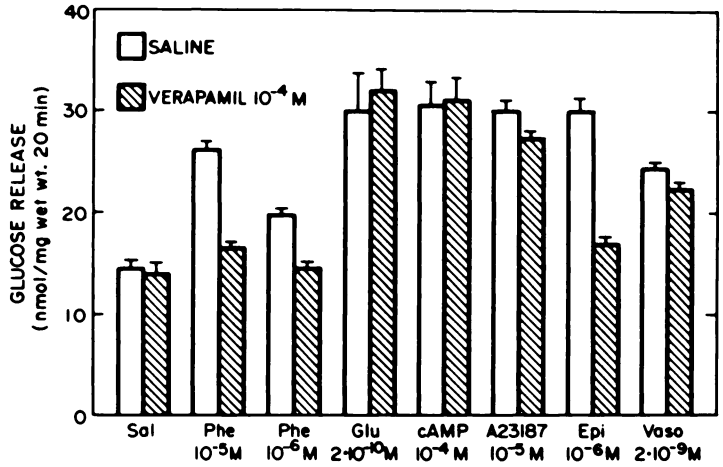


FIG. 1. *Effect of verapamil on glycogenolysis in hepatocytes incubated with phenylephrine (Phe), glucagon (Glu), exogenous cAMP, A23187, epinephrine plus propranolol $10^{-5}$ M (Epi), and vasopressin (Vaso)*

Hepatocytes were incubated for 15 min prior to the addition of hormone or other agents and then for 20 min with the additions shown. Data are means ± SEM from a typical experiment performed in triplicate.

teristics of α-adrenergic receptors. Figure 2 shows that verapamil inhibited the binding of [$^3$H]epinephrine with $2 \times 10^{-5}$ M producing half-maximal inhibition. Phenoxybenzamine, an established α-blocker, produced the same maximal inhibition of [$^3$H]epinephrine as did verapamil, although phenoxybenzamine was more potent as expected from previous results (3). The β-blocker sotalol was completely ineffective which is to be expected since propranolol was present.

*Effects of verapamil on epinephrine-induced $^{45}$Ca efflux from hepatocytes.* Epinephrine and phenylephrine, acting through α-receptors, stimulate $^{45}$Ca efflux from hepatocytes preloaded with the radionuclide (2, 4). Figure 3 shows that verapamil had a slight effect on basal $^{45}$Ca efflux, but inhibited the efflux induced by $4 \times 10^{-8}$ M epinephrine in accord with its postulated α-antagonistic action. Its potency in this system is similar to its potency as an inhibitor of epinephrine binding to liver membranes. As expected, phenoxybenzamine was more potent and sotalol was ineffective. Although not shown, the three agents had similar effects on epinephrine-induced phosphorylase activation to those seen on $^{45}$Ca efflux.

*Effects of verapamil on epinephrine-induced changes in calcium and phosphorylase a levels in hepatocytes.* Previous experiments (4) have shown that, in addition to promoting $^{45}$Ca efflux from pre-loaded hepatocytes, epinephrine also causes a concurrent loss of chemical calcium, and that this is also mediated by α-receptors. Figure 4 illustrates that verapamil at concentrations higher than $10^{-5}$ M reversed the loss of calcium induced by $4 \times 10^{-8}$ M epinephrine. Coincident with this reversal was an inhibition of phosphorylase activation. The potency of verapamil as an inhibitor of

TABLE 1

*Failure of increased medium $Ca^{2+}$ concentration to reverse verapamil inhibition of phenylephrine-stimulated glycogenolysis*

Hepatocytes were incubated for 20 min in media containing the $Ca^{2+}$ concentrations shown in the presence or absence of $10^{-6}$ M phenylephrine or $10^{-4}$ M verapamil. Results from a single experiment performed in triplicate are shown.

| $Ca^{2+}$ concentration | Saline | Glucose release | |
|---|---|---|---|
| | | Phenylephrine | Phenylephrine + verapamil |
| *mM* | | *nmol/mg wet wt. 20 min* | |
| 2.65 | 10 ± 1 | 15 ± 1 | 9 ± 2 |
| 5 | 10 ± 1 | 16 ± 1 | 8 ± 1 |
| 10 | 10 ± 1 | 14 ± 2 | 7 ± 1 |

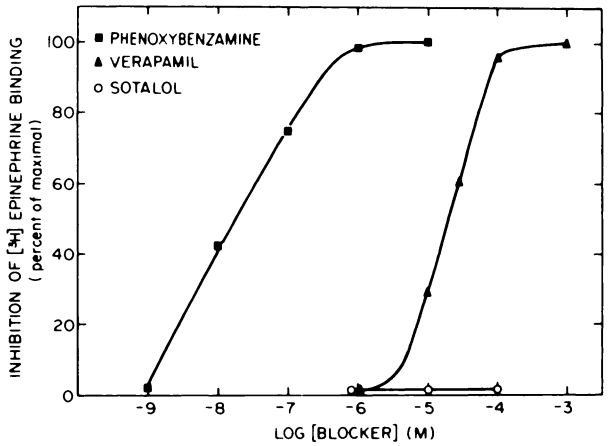


FIG. 2. *Inhibition of (±)[$^3$H]epinephrine binding to rat liver plasma membranes by phenoxybenzamine, verapamil and sotalol*

Rat liver plasma membranes (approx. 2 mg protein/ml) were incubated with $4 \times 10^{-8}$ M (±)[$^3$H]epinephrine (the concentration producing half-maximal binding) and with increasing concentrations of phenoxybenzamine, verapamil and sotalol. Binding assays were performed as described under MATERIALS AND METHODS. Results are expressed as percent of the amount of (±)[$^3$H]epinephrine bound in the absence of any blocking agent. Each value is the mean of triplicate determinations and the experiment is representative of three.

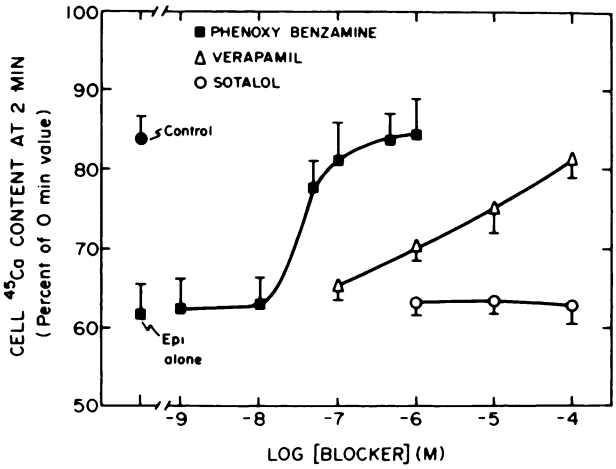


FIG. 3. *Effect of phenoxybenzamine, verapamil and sotalol on epinephrine-stimulated $^{45}$Ca release from hepatocytes*

Hepatocytes were loaded with $^{45}$Ca as described before (2, 4). The cells were washed and incubated in normal buffer containing no $^{45}$Ca, $4 \times 10^{-8}$ M epinephrine and the concentrations of blockers shown. The cell content of $^{45}$Ca was measured at zero time and two minutes later. Results are expressed as percent of zero time value and are from a representative experiment performed in triplicate. Control incubations with blockers alone gave the following $^{45}$Ca content values (expressed as percent of 0 min value); phenoxybenzamine $10^{-6}$ M, 86 ± 1; verapamil $10^{-4}$ M, 79 ± 2; and sotalol $10^{-4}$ M, 86 ± 2.

these parameters was very similar to that of its inhibition of plasma membrane epinephrine binding (Fig. 4, cf. Fig. 2). Verapamil ($10^{-4}$ M) added alone had no consistent effects on cell calcium and phosphorylase *a* levels (Figs. 4–6). As expected from Fig. 1, verapamil inhibited the effects of phenylephrine on calcium content and phosphorylase *a*, but not those of vasopressin and glucagon (Fig. 5).

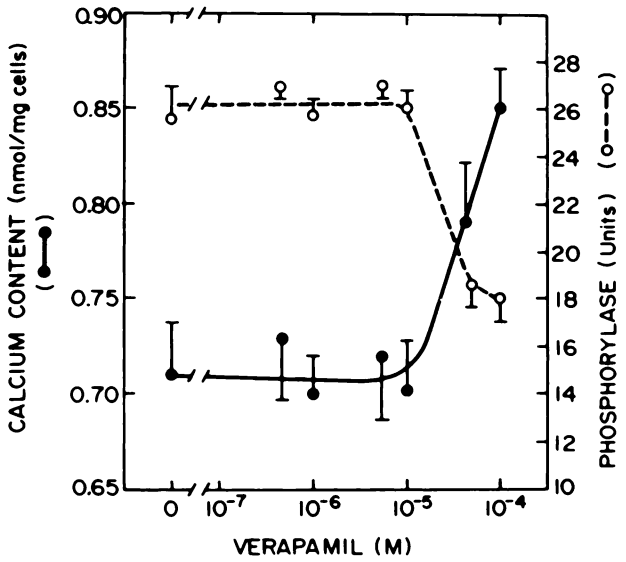


FIG. 4. *Effect of verapamil on epinephrine-stimulated calcium efflux and phosphorylase a activity in hepatocytes*

Five minutes after the addition of epinephrine ($4 \times 10^{-8}$ M) and various concentrations of verapamil, samples (0.5 ml each) were removed for measurement of calcium content and phosphorylase *a* levels as described under MATERIALS AND METHODS. Units of phosphorylase *a* were those defined previously (9). Phosphorylase *a* levels in saline and verapamil ($10^{-4}$ M) alone, incubations were 13.8 ± 0.9 units and 17.5 ± 0.5 units, respectively, while calcium contents in nmole per mg of cells were 0.88 ± 0.02 and 0.85 ± 0.013, respectively.

The competitive nature of verapamil inhibition of α-adrenergic stimulation of glycogenolysis is shown in Fig. 6. Increasing the concentration of verapamil caused parallel rightward shifts of the phenylephrine dose-response curves between $10^{-6}$ M and $5 \times 10^{-6}$ M, although there was an apparent depression of the maximum response. A Schild plot of the data gives a $pA_2$ of $2.5 \times 10^{-5}$ M and a slope of 1. This is consistent with apparent competitive inhibition, although a small non-competitive component cannot be ruled out, especially since high concentrations of verapamil ($10^{-4}$ M and above) not only possess α-adrenergic blocking action, but also exert a slight agonist activity (e.g., Figs. 4, 5 and 7).

*Effects of verapamil on A23187-induced calcium efflux and influx in hepatocytes.* The preceding experiments provided no evidence that verapamil altered net calcium efflux from hepatocytes, but did not test whether the agent affected calcium influx. Recent findings in mammalian sperm (16) and hepatocytes (4) indicate that the divalent cation ionophore A23187 can produce either net calcium efflux or influx depending on its concentration. Thus low concentrations cause an efflux due presumably to release of $Ca^{2+}$ from mitochondria, whereas high concentrations cause an influx due to $Ca^{2+}$ leakage across the plasma membrane (Ref. 4 and 16 and Fig. 7). The ability of verapamil to inhibit $Ca^{2+}$ uptake was therefore measured using different concentrations of A23187. Figure 7 (left panel) shows that verapamil ($10^{-4}$ M and $5 \times 10^{-4}$ M) was without effect on the loss of cell calcium induced by $10^{-7}$ M and $5 \times 10^{-7}$ M ionophore, but inhibited the accumulation induced by concentrations between $10^{-6}$ M and $10^{-5}$ M. Phosphorylase activation was minimally affected by $10^{-4}$ M verapamil at any concentration of ionophore (Fig. 7, right panel), perhaps because the efflux of $Ca^{2+}$ from mitochondria was the major contributor to cytosolic $Ca^{2+}$ at all concentrations (4). The higher concentration of verapamil ($5 \times 10^{-4}$ M) added alone markedly activated phosphorylase (data not shown).

*Effects of verapamil on calcium uptake by hepatocytes depleted of calcium.* Al-

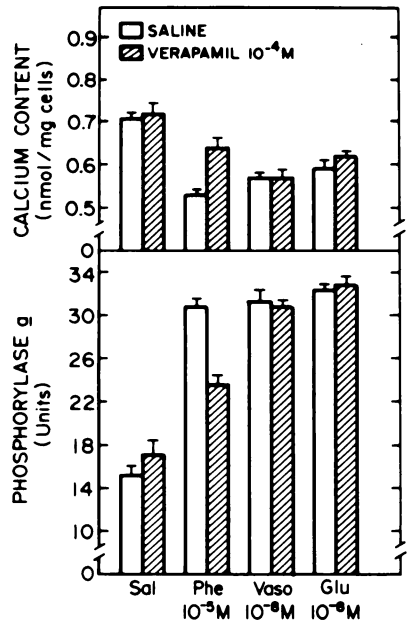


FIG. 5. *Effect of verapamil ($10^{-4}$ M) on phenylephrine (Phe)-, vasopressin (Vaso)- and glucagon (Glu)-stimulated calcium efflux and phosphorylase activation*

Five minutes after the addition of each agent with or without verapamil, samples were removed for the determination of cell calcium content and phosphorylase *a* activity.

though verapamil inhibited A23187-induced calcium uptake, this does not mean that the antagonist can block the movement of cation through the natural channels of the plasma membrane. In fact, verapamil could have acted by competing with $Ca^{2+}$ ions for the binding site of A23187 (17). The ability of verapamil to block the uptake of calcium into calcium-depleted hepatocytes was therefore examined. Figure 8 shows that increasing the calcium concentration in the medium results in an increase in the intracellular level of calcium measured at 1 min. The inclusion of verapamil $10^{-4}$ M or $5 \times 10^{-4}$ M did not produce any consistent effect on calcium uptake at any concentration of calcium tested. These results suggest that verapamil is ineffective at inhibiting the uptake of calcium through the hepatocyte plasma membrane channels.

## DISCUSSION

The present results raise doubts about the usefulness of verapamil as a calcium antagonist in tissues other than cardiac and smooth muscle. In addition, they raise questions about the interpretation of the results of experiments in which catecholamines have been used together with verapamil. Previous workers have pointed to the fact that the agent produces effects other than those attributable to altered calcium flux (14, 18–20). It is also of interest to note that when verapamil was first described by Haas and Haertfelder (19), it was classified as a $\beta$-adrenergic blocker.

Based on its effects on [$^3$H]epinephrine binding to liver plasma membranes and on epinephrine- and phenylephrine-stimulated glycogenolysis, phosphorylase activation, $^{45}Ca$ efflux and calcium loss on hepatocytes, verapamil can be characterized as an $\alpha$-adrenergic blocker. If verapamil and its methoxy derivative D600 exert a similar action in other tissues, many results from a variety of previous studies may be explained. This is because the precise locus of interaction between verapamil and many subcellular structures, particularly plasma membranes, was not characterized in these tissues. Examples are: a) the inhibition by verapamil ($10^{-5}$ M) of norepinephrine ($10^{-6}$ M)-induced contraction of rabbit pulmonary artery and cat nictilating membrane (13); b) the inhibition by D600 ($10^{-7}$ M) of the positive inotropic effect of phenylephrine ($10^{-7}$–$10^{-4}$ M) in rabbit papillary muscle (21), of the effects of norepinephrine ($10^{-9}$–$10^{-5}$ M) on the contraction of guinea pig atrium (22) and of rabbit aorta and mesenteric artery (23); c) the inhibition by verapamil ($10^{-5}$ M) of norepinehrine ($2 \times 10^{-6}$ M)-induced contraction of guinea pig portal vein (24); d) the abolition by verapamil ($5 \times 10^{-5}$ M) of phenylephrine ($3 \times 10^{-5}$ M)-induced contractions of rat vas deferens (25); e) the inhibition by verapamil (18 $\mu$g/kg) of arrhythmias induced by norepinephrine (5 $\mu$g/kg) in dog myocardium (26); and f) the inhibition by D600 ($10^{-4}$ M) of phenylephrine ($2.5 \times 10^{-6}$ M)-induced $K^+$ release, cGMP accumulation, and amylase release from parotid cells, but not of $K^+$ release

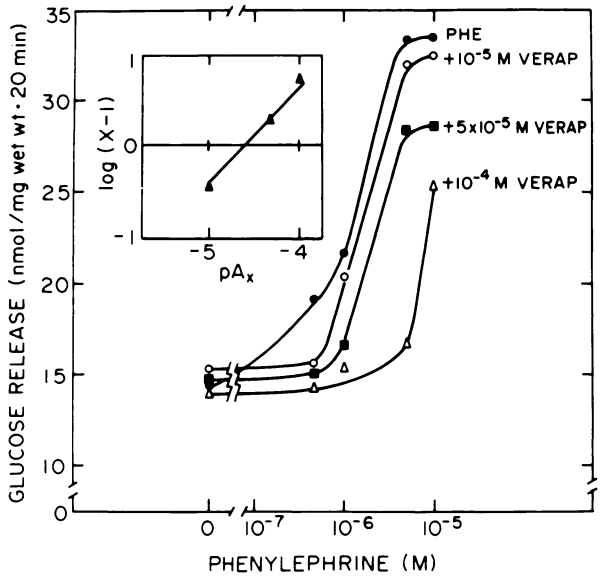


FIG. 6. *Inhibition of phenylephrine-stimulated glycogenolysis in hepatocytes by verapamil*

Twenty minutes after the addition of various doses of phenylephrine (PHE) and verapamil (VERAP), 0.5 ml samples were removed for measurement of glucose. A Schild plot (15) (insert) of these data is shown. Half-maximal stimulatory concentrations of phenylephrine were determined by probit plots. The $pA_2$ is the intercept of the line with the abscissa and $\times$ is the ratio of the concentration of phenylephrine causing half-maximal glucose release in the presence and absence of verapamil concentrations shown on the abscissa.

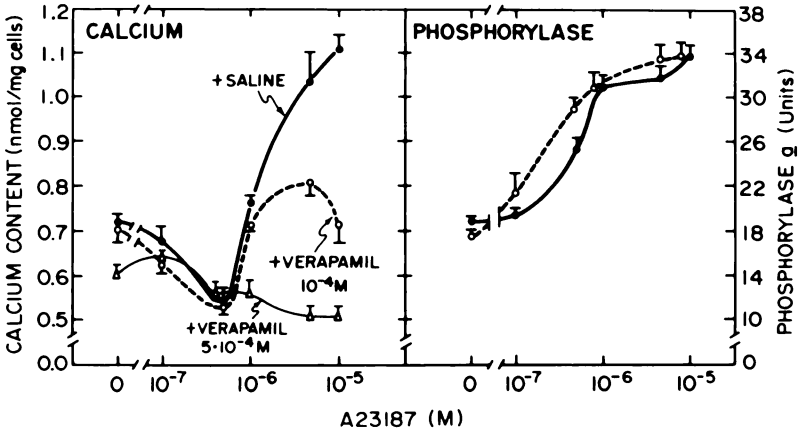


FIG. 7. *Effect of verapamil on the dose responses of A23187 on cell calcium content and phosphorylase activation in hepatocytes*

Cells were incubated with verapamil ($10^{-4}$ M or $5 \times 10^{-4}$ M) and A23187 ranging in concentration from $10^{-7}$ to $10^{-5}$ M. Five minutes later samples were removed for determination of cell calcium and phosphorylase *a* levels. Control incubations contained 1% (v/v) dimethyl sulfoxide, the solvent used to dissolve A23187.

produced by substance P and A23187 (27). In the majority of these studies no calcium flux measurements were made to verify that verapamil was in fact inhibiting transmembrane calcium movements.

The present study failed to provide evidence that verapamil inhibited either calcium influx or efflux in isolated hepatocytes either in the basal state or in the presence of agents mobilizing intracellular calcium

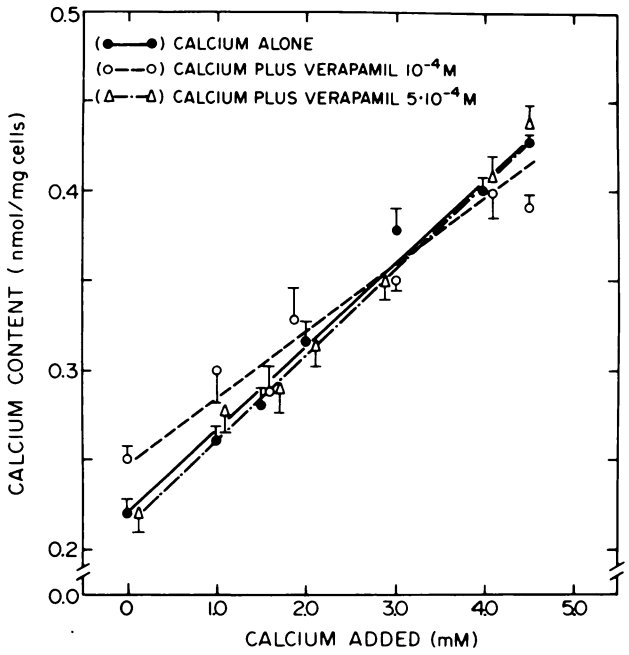


FIG. 8. *Effect of calcium addition on calcium content of calcium-depleted cells in the presence and absence of verapamil*

Cells were washed and incubated in Ca-free medium containing 1 mM EGTA as previously described (2). Calcium was added to the cells at the concentrations shown, in the presence and absence of verapamil $10^{-4}$ M and $5 \times 10^{-4}$ M and 1 min later the cell calcium content was measured (4). Data are means ± SEM from a typical experiment performed in triplicate.

such as vasopressin or high concentrations of glucagon. Although verapamil blocked calcium uptake induced by high concentrations of A23187, this could be due to its displacement of calcium from the binding site of the ionophore. This possibility is suggested by the fact that verapamil inhibits A23187-mediated calcium translocation from an aqueous to an organic phase in an artificial model (17).

In summary, the present findings illustrate that caution should be used when pharmacological agents with properties characterized for one tissue are used in studies of other tissues. To our knowledge, the ability of verapamil to inhibit transmembrane $Ca^{2+}$ flux has only been demonstrated convincingly in cardiac muscle and it would appear to have other unrelated, physiologically important, effects in this tissue.

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