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STUDIES WITH VERAPAMIL AND NIFEDIPINE PROVIDE EVIDENCE FOR THE PRESENCE IN THE LIVER CELL PLASMA MEMBRANE OF TWO TYPES OF Ca^{2+} INFLOW TRANSPORTER WHICH ARE DISSIMILAR TO POTENTIAL-OPERATED Ca^{2+} CHANNELS

BERNARD P. HUGHES, SUSAN E. MILTON, GREGORY J. BARRITT and AMANDA M. AULD
Department of Biochemistry and Chemical Pathology, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia

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Abstract—The addition of 500 μM verapamil or nifedipine to isolated hepatocytes incubated in the presence of 1.3 mM Ca^{2+} caused 20% inhibition of Ca^{2+} inflow as measured by the initial rate of $^{45}Ca^{2+}$ exchange. No stimulation of $^{45}Ca^{2+}$ exchange was observed in the presence of the Ca^{2+} agonist CGP 28392. An increase in the concentration of extracellular K^+ from 6 to 60 mM (to depolarize the plasma membrane) increased the initial rate of $^{45}Ca^{2+}$ exchange by 30%. In the presence of 60 mM K^+, 400 μM verapamil inhibited the initiate rate of $^{45}Ca^{2+}$ exchange by 50%. Verapamil and nifedipine completely inhibited vasopressin-induced Ca^{2+} inflow as determined by measurement of the initial rate of $^{45}Ca^{2+}$ exchange and of glycogen phosphorylase *a* activity. This effect of verapamil was completely reversed by increasing the extracellular concentration of Ca^{2+}. The concentrations of Ca^{2+} antagonist which gave 50% inhibition of vasopressin- or K^+-stimulated Ca^{2+} inflow were in the range 50–100 μM, about 50-fold greater than the concentration which gave 50% inhibition of the beating of electrically-stimulated myocardial muscle cells. In the absence of vasopressin, verapamil caused a transient increase in glycogen phosphorylase *a* activity by a process which is largely independent of Ca^{2+}. It is concluded that verapamil and nifedipine inhibit the transport of Ca^{2+} across the hepatocyte plasma membrane through a putative Ca^{2+} transporter which is activated by vasopressin and which differs in nature from potential-operated Ca^{2+} channels in excitable cells and from the Ca^{2+} transporter present in hepatocytes in the absence of hormone.

Changes in the cytoplasmic Ca^{2+} concentration act as an intracellular messenger in the action of a number of hormones on the liver cell [1, 2] (reviewed by Exton [3]). The transport of Ca^{2+} across the liver cell plasma membrane plays a major role in regulation of the concentration of free Ca^{2+} in the cytoplasm [4–6]. Moreover, evidence that α-adrenergic agonists, vasopressin and angiotensin increase the rate of plasma membrane Ca^{2+} inflow has been obtained [1, 2, 5–12].

In the absence of hormones, the estimated flux of Ca^{2+} across the liver cell plasma membrane, expressed per unit area [7], is larger than that observed for most other cell types [13]. The nature of the transporters which catalyse the inflow of Ca^{2+} is poorly understood. Some evidence for the presence of specific Ca^{2+} channels or transporters [7–9, 14], and transporters which can be described as receptor-activated channels [7] has been obtained.

In excitable cells, including those of myocardial and smooth muscle, the major proportion of Ca^{2+} entry across the plasma membrane is catalysed by potential-operated Ca^{2+} channels (reviewed in refs 15–18). One of the characteristics of these channels is inhibition or activation by low concentrations of Ca^{2+} antagonists (including verapamil, nifedipine and diltiazem) and agonists (including Bay K8644 and CGP 28392), respectively (reviewed in refs 16, 17, 19–22).

The response of plasma membrane Ca^{2+} inflow in the liver cell to Ca^{2+} antagonists and agonists has not been well characterized. While some studies with isolated hepatocytes or perfused livers have been performed [9, 23, 24] the results have not permitted definite conclusions to be reached about the nature of the susceptibility of Ca^{2+} inflow transporters in the liver cell plasma membrane to the action of this group of drugs. Furthermore, the possibility that the observed effects of Ca^{2+} antagonists on the perfused liver [24] are due to the interaction of the drugs with non-parenchymal cells cannot be excluded.

The aim of the present experiments was to determine whether Ca^{2+} inflow transporters in the hepatocyte plasma membrane are all of one type and whether these transporters have properties similar to those of the potential-operated Ca^{2+} channels present in excitable cells. The susceptibility to inhibition by the Ca^{2+} antagonists, verapamil and nifedipine, and to activation by the Ca^{2+} agonist CGP 28392 [22], were tested in the absence and presence of the hormone agonist, vasopressin. Dose–response curves for the effect of verapamil on Ca^{2+} inflow to the liver cell were compared with those for the effects of the drug on potential-operated Ca^{2+}

channels in isolated myocardial muscle cells, since it was considered important to make a comparison of the properties of liver cells with those of excitable cells in the same laboratory.

MATERIALS AND METHODS

Materials. Nifedipine was obtained from the Sigma Chemical Co. (St. Louis, MO). Verapamil and D600 were kindly provided by the Knoll AG and CGP 28392 by Ciba-Geigy Ltd. (Basel, Switzerland). Sodium β-glycerophosphate, caffeine and glycogen (Type VII from Mussel *Mytilus edulis*) were purchased from the Sigma Chemical Co. (St. Louis, MO); glucose-1-phosphate from Boehringer Mannheim Australia Pty. Ltd. (North Ryde, N.S.W.); and [U-^{14}C]glucose-1-phosphate and the acetoxymethylester of quin2 from Amersham Australia Pty. Ltd. (Sydney, N.S.W.). All other materials were of the highest grade available and were purchased from the sources described previously [7, 25].

Methods. The preparation of isolated hepatocytes from fed rats, incubation of hepatocytes, and measurement of the initial rates of $^{45}Ca^{2+}$ exchange were conducted as described previously [7]. The incubation medium contained, in a final volume of 6.0 ml, 117 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 20 mM 2-([hydroxy-1,1,bis(hydroxymethyl)ethyl]amino)-ethanesulphonic acid (Tes)-KOH, 1.3 mM $CaCl_2$; hepatocytes (30 mg wet wt. (2×10^6 cells) per ml) and other additions as indicated. The final pH was 7.4.

Some $^{45}Ca^{2+}$ exchange experiments were conducted in a medium enriched in K^+ in order to depolarize the plasma membrane [24, 26, 27]. For these experiments hepatocytes were prepared in the normal manner and suspended in the incubation medium described above (in the absence of Ca^{2+}). The cells were then washed, re-suspended and incubated in a medium of similar composition except that the Tes was omitted and the concentrations of K^+ and Na^+ were 63 and 85 mM, respectively.

Glycogen phosphorylase *a* activity was estimated

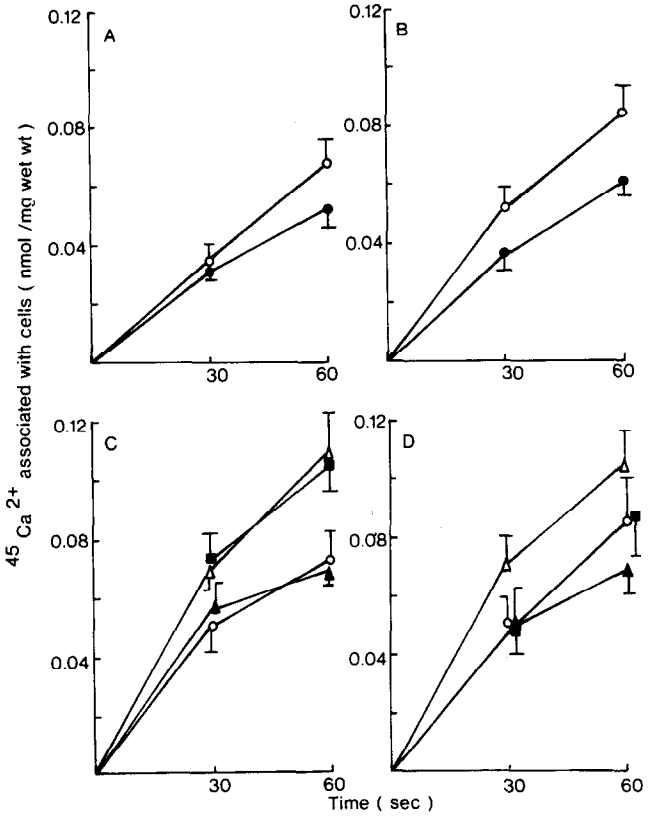


Fig. 1. Effects of the Ca^{2+} antagonists, nifedipine (A, C) and verapamil (B, D) on the initial rate of $^{45}Ca^{2+}$ exchange in the absence (A, B) and presence (C, D) of vasopressin. Initial rates of $^{45}Ca^{2+}$ exchange in the absence of any further addition (control) (○) and in the presence of 50 μM nifedipine or 40 μM verapamil (●), vasopressin (△), vasopressin plus 50 (■) or 500 μM (▲) nifedipine, or vasopressin plus 40 (■) or 400 (▲) μM verapamil, were measured as described in Materials and Methods. Hepatocytes were incubated for 15 min in the presence of 1.3 mM Ca^{2+} before addition of tracer amounts of $^{45}Ca^{2+}$. In the absence of vasopressin (A and B) the Ca^{2+} antagonist was added 5 min before the $^{45}Ca^{2+}$. In the presence of vasopressin (C and D) the hormone and Ca^{2+} antagonist were added at 5 and 10 min, respectively, before addition of $^{45}Ca^{2+}$. The results are the means ± SE of two determinations on each of 2–6 cell preparations.

as described by Hutson *et al.* [28]. The activity of the enzyme is expressed as μmol [^{14}C]glucose-1-phosphate incorporated into glycogen per min per g wet wt. of cells (1 unit).

The loading of hepatocytes with quin2 by incubation with the acetoxymethylester of quin2 and measurement of intracellular Ca^{2+} by quin2 fluorescence were conducted as described by Charest *et al.* [29], using an Aminco-Bowman spectrofluorimeter (SPF 74, American Instrument Company, Silver Spring, MD).

The preparation of suspensions of isolated ventricular myocardial muscle cells from rat hearts and measurement of the beating of isolated myocardial muscle cells in response to electrical stimulation were conducted as described previously [25].

Verapamil and nifedipine were initially dissolved in dimethylsulphoxide. The final concentration of dimethylsulphoxide was less than 1% (v/v). Control incubations received equal amounts of dimethylsulphoxide.

RESULTS

Rates of Ca^{2+} inflow across the plasma membrane were estimated by measuring the rate of uptake of $^{45}Ca^{2+}$ (added in trace amounts) to hepatocytes incubated under steady-state conditions at 1.3 mM extracellular Ca^{2+}. Previous compartmental analysis of kinetic data has indicated that the initial rate of $^{45}Ca^{2+}$ uptake measured under these conditions is a reliable estimate of Ca^{2+} flux from the medium to the cell [5, 7].

In the absence of vasopressin, nifedipine (50 μM) and verapamil (40 μM) caused a small inhibition of the initial rate of $^{45}Ca^{2+}$ exchange (Fig. 1a, b). Dose–response curves showed only partial inhibition at the highest concentrations of Ca^{2+} antagonist tested (Fig. 2). No stimulation of the initial rate of $^{45}Ca^{2+}$ exchange was observed in the presence of 4, 40 or 400 μM Ca^{2+} agonist CGP 28392 (results not shown).

When the concentration of extracellular K^+ was increased from 6 to 63 mM (high K^+ buffer) in order to depolarize the plasma membrane [24, 26, 27] an increase of 30% in the initial rate of $^{45}Ca^{2+}$ exchange was observed (Fig. 3, inset). In experiments in which the $^{45}Ca^{2+}$ exchange curves were measured over a period of 45 min the curve obtained for cells incubated in high K^+ buffer was slightly greater (10%) than that observed at normal K^+ (results not shown). In the presence of high K^+ buffer, the inhibition by verapamil was greater than that observed at the normal extracellular K^+ concentration (Fig. 3 cf. Fig. 2). However, these concentrations of verapamil are at least an order of magnitude higher than those which inhibit the beating of isolated myocardial muscle cells depolarized by electrical stimulation (Fig. 4) or the increase in myoplasmic free Ca^{2+} induced by increases in extracellular K^+ (results not shown).

In the presence of vasopressin, nifedipine and verapamil inhibited the hormone-stimulated initial

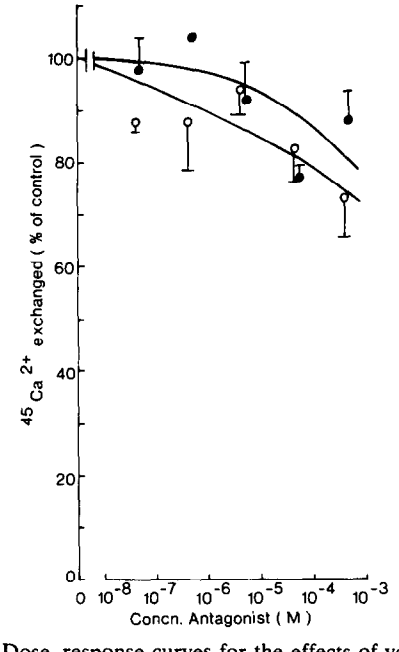


Fig. 2. Dose–response curves for the effects of verapamil and nifedipine on the initial rate of $^{45}Ca^{2+}$ exchange. Amounts of $^{45}Ca^{2+}$ exchanged were measured at 60 sec after the addition of tracer amounts of $^{45}Ca^{2+}$ in the presence or absence of verapamil (○) or nifedipine (●) as described in Materials and Methods and the legend of Fig. 1. The amount of $^{45}Ca^{2+}$ exchanged in the presence of the Ca^{2+} antagonist is expressed as a percentage of that observed in the absence of Ca^{2+} antagonist. The results are the mean (N = 2) or the mean ± SE of determinations with 3–7 separate cell preparations.

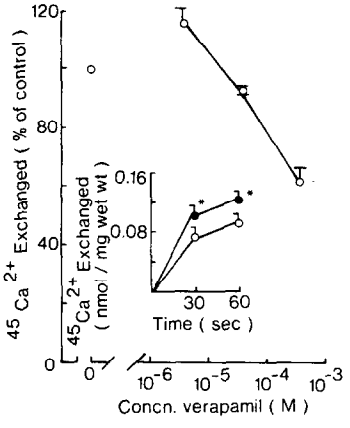
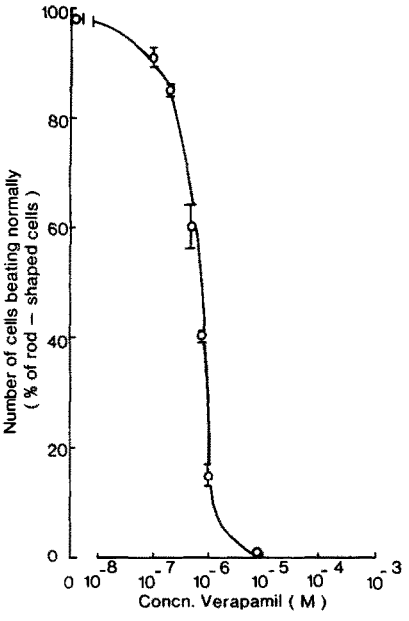


Fig. 3. Inhibition by verapamil of $^{45}Ca^{2+}$ exchange measured in the presence of elevated concentrations of K^+. The amount of $^{45}Ca^{2+}$ exchanged at 60 sec was measured in a medium which contained 63 mM K^+ and 85 mM Na^+, as described in Materials and Methods. The amounts of $^{45}Ca^{2+}$ exchanged in the presence of verapamil are expressed as a percentage of the value obtained in the absence of the Ca^{2+} antagonist. The inset shows plots of the amount of $^{45}Ca^{2+}$ associated with the cells as a function of time for hepatocytes incubated under standard conditions (6 mM K^+ and 142 mM Na^+) (○) or in the presence of 63 mM K^+ and 85 mM Na^+ (●). The results are the means ± SE of experiments conducted with 5 separate cell preparations (inset) or the means of 2 experiments conducted in duplicate with two separate cell preparations. The value of P, determined using Student's *t*-test for paired samples, is: $^*P < 0.05$.



rate of exchange (Figs. 1c, d and 5a). The effects of the Ca^{2+} antagonists on Ca^{2+} inflow were also tested by measuring the ability of vasopressin to increase the activity of glycogen phosphorylase *a*. The addition of verapamil alone to hepatocytes incubated in the presence of 0 or 1.3 mM extracellular Ca^{2+} caused a transient increase in glycogen phosphorylase activity (Fig. 6). This did not impair subsequent activation of glycogen phosphorylase by addition of vasopressin at 11 min (results not shown).

Fig. 4. Dose–response curve for the effect of verapamil on the contraction of isolated myocardial muscle cells. Measurement of the number of rod-shaped cells which beat in response to electrical-stimulation was conducted as described in Materials and Methods. The number of rod-shaped cells which beat in response to electrical stimulation is expressed as a percentage of the total number of rod-shaped cells. The number of rod-shaped cells was 53 ± 4% (N = 4; mean ± SE) of the total number of cells present. The results are the means ± SE of two determinations on each of two separate cell preparations.

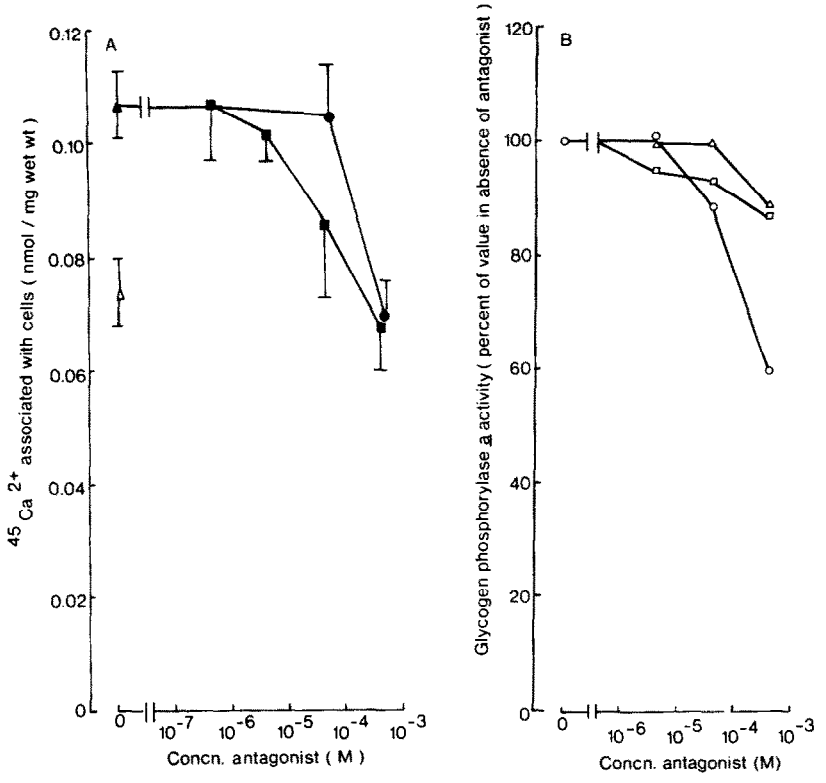


Fig. 5. Dose–response curves for the effects of Ca^{2+} antagonists on the vasopressin-stimulated initial rate of $^{45}Ca^{2+}$ exchange (A) and the activity of glycogen phosphorylase *a* (B). The amount of $^{45}Ca^{2+}$ exchanged (A) was measured at 60 sec after the addition of tracer amounts of $^{45}Ca^{2+}$ in the absence of vasopressin (△), in presence of vasopressin (▲), and in the presence of vasopressin and verapamil (■) or nifedipine (●) as described in Materials and Methods. The results are the means ± SE of two determinations on each of 7 (no Ca^{2+} antagonist present), 3 (nifedipine present) or 2 (verapamil present) separate cell preparations. Glycogen phosphorylase *a* activity (B) was measured at 5 min after the addition of vasopressin (20 nM) in the presence of verapamil (○), nifedipine (□) or D600 (△) as described in the legend of Fig. 7. Enzyme activity in cells incubated in the presence of a given antagonist is expressed as a percentage of that present in the absence of antagonist. The results are the means of experiments conducted with two separate cell preparations (verapamil and nifedipine) and of one experiment (D600).

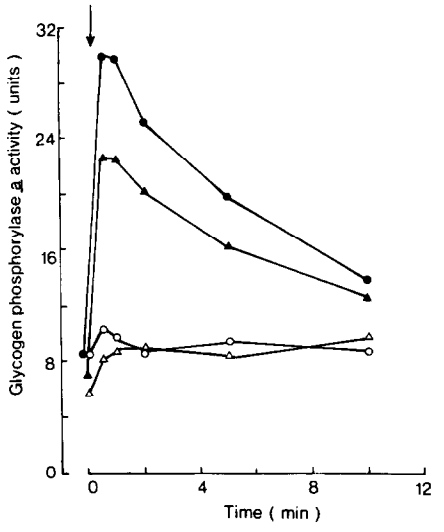


Fig. 6. Effect of verapamil on the activity of glycogen phosphorylase *a* in the absence of hormone. Hepatocytes were incubated for 25 min in the presence of 1.3 mM Ca^{2+} (circles) or in the absence of added Ca^{2+} (triangles) before the addition of 400 μM verapamil (closed symbols) or dimethylsulphoxide (open symbols) at 0 min (indicated by the arrow). Glycogen phosphorylase *a* activity was measured as described in Materials and Methods. The results are the means of experiments conducted with two separate cell preparations.

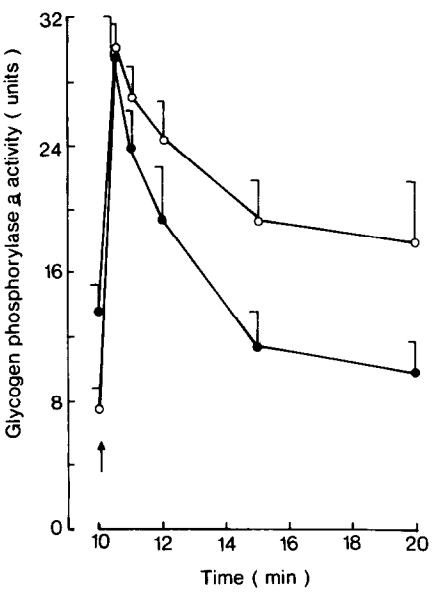


Fig. 7. Effect of verapamil on the activation by vasopressin of glycogen phosphorylase *a* in hepatocytes incubated at 1.3 mM Ca^{2+}. Cells were incubated for 15 min before the addition of 400 μM verapamil (●) or dimethylsulphoxide (○) (t = 0 min on the graph). After 10 min, 20 nM vasopressin was added (indicated by the arrow). The activity of glycogen phosphorylase *a* was measured as described in Materials and Methods. The results are the means of experiments conducted in duplicate with two separate cell preparations.

No change in intracellular free Ca^{2+} concentration, monitored using quin2, was detected following the addition of verapamil under these conditions (results not shown). At 1.3 mM extracellular Ca^{2+}, 40 μM verapamil had no effect while maximal increase in phosphorylase activity was observed with 400 μM Ca^{2+} antagonist (results not shown). Addition of 400 μM CGP 28392 to hepatocytes incubated at 1.3 mM Ca^{2+} also increased the activity of glycogen phosphorylase to 15.5 units at 5 min after addition of the drug compared with a value of 8.4 units (means of 2 determinations) in the absence of the drug.

For hepatocytes incubated at 1.3 mM Ca^{2+}, the addition of verapamil 10 min before subsequent addition of vasopressin (i.e. after completion of the transient effect induced by verapamil alone (Fig. 6)) did not affect the ability of vasopressin to increase glycogen phosphorylase at 0.5 min, but decreased the activity of the enzyme observed at subsequent times (Fig. 7). The plot obtained in the presence of 1.3 mM Ca^{2+}, vasopressin and verapamil (Fig. 7) is similar to that obtained in an identical experiment conducted in the presence of vasopressin but in the absence of added Ca^{2+} and verapamil (results not shown). Inhibition of hormone-stimulated glycogen phosphorylase activity at 5 and 10 min is consistent with an inhibition by verapamil of Ca^{2+} inflow across the plasma membrane [10–12, 30] and hence the resulting increase in intracellular free Ca^{2+} concentration. The concentration of verapamil which gave half-maximal inhibition (Fig. 5b) was similar to that which gave half-maximal inhibition of vasopressin-stimulated $^{45}Ca^{2+}$ exchange. Some inhibition was also observed in the presence of D600 and nifedipine (Fig. 5b). The inhibition by verapamil was overcome by increasing the concentration of extracellular Ca^{2+} (Table 1).

The addition of 1.3 mM Ca^{2+} to hepatocytes previously incubated in the absence of Ca^{2+} increased glycogen phosphorylase activity (Fig. 8). A much greater activation of phosphorylase was observed in the presence of vasopressin. This is presumably due, at least in part, to the activation of plasma membrane Ca^{2+} transporters by vasopressin. The activation of glycogen phosphorylase by Ca^{2+} observed in the presence of vasopressin was completely inhibited by 400 μM verapamil (Fig. 8). It was not possible to determine the effect of verapamil on the increase in glycogen phosphorylase induced by Ca^{2+} in the absence of vasopressin since, as discussed above, verapamil alone induces an increase in glycogen phosphorylase activity (Fig. 6). Measurement of the intracellular free Ca^{2+} concentration in quin2-loaded cells confirmed that in the presence of vasopressin the increase in intracellular free Ca^{2+} concentration induced by addition of Ca^{2+} is completely inhibited by verapamil (results not shown).

DISCUSSION

The conclusion that verapamil and nifedipine inhibit the transport of Ca^{2+} across the hepatocyte plasma membrane through a Ca^{2+} transporter activated by vasopressin is consistent with the following observations. (1) The Ca^{2+} antagonists inhibit vasopressin-stimulated $^{45}Ca^{2+}$ exchange, and the elevated glycogen phosphorylase activity induced by

Table 1. Effect of increasing extracellular Ca^{2+} concentrations on the inhibition by verapamil of vasopressin-stimulated glycogen phosphorylase *a* activity*

Extracellular Ca^{2+} concentration (mM)	Activity of glycogen phosphorylase *a* (units)	
	Dimethylsulphoxide	Verapamil
1.3	22	12
1.9	22	16
4.0	22	20
7.4	20	21
10.6	22	21

* Hepatocytes were incubated in the presence of the indicated concentration of Ca^{2+} as described in the legend of Fig. 7. Glycogen phosphorylase *a* activity was measured at 10 min after addition of vasopressin. The data are the means of the results obtained with two separate cell preparations. Similar results were obtained when glycogen phosphorylase activity was measured 5 min after addition of vasopressin.

treatment of hepatocytes incubated at 1.3 mM Ca^{2+} with vasopressin for 5 or 10 min. Other studies have provided evidence which indicates that the latter response to vasopressin involves the inflow of Ca^{2+} across the plasma membrane [10–12, 30]. (2) Verapamil inhibited the increase in glycogen phosphorylase activity which follows the addition of Ca^{2+} to Ca^{2+}-deprived cells incubated in the presence of vasopressin. (3) High concentrations of Ca^{2+} reversed the inhibitory effect of verapamil on glycogen phosphorylase activity.

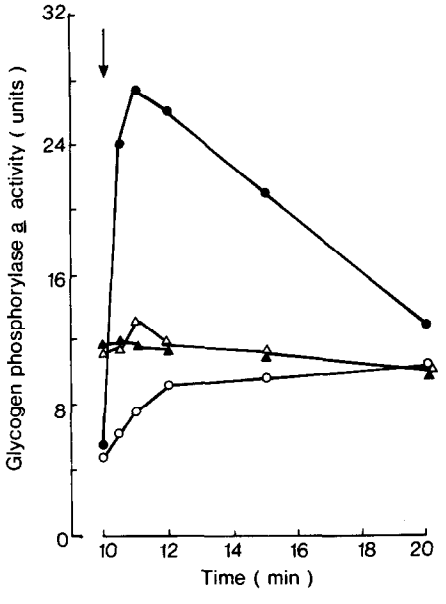


Fig. 8. Inhibition by verapamil of the activation of glycogen phosphorylase *a* following the addition of 1.3 mM Ca^{2+} to Ca^{2+}-deprived hepatocytes incubated in the presence of vasopressin. Cells were incubated in the absence of added Ca^{2+} (Ca^{2+}-deprived cells) for 20 min before the addition of dimethylsulphoxide (circles) or 400 μM verapamil (triangles) (t = 0 min on the graph). After 5 min, saline (open symbols) or vasopressin (closed symbols) were added. After a further 5 min (t = 10 min on the graph) 1.3 mM Ca^{2+} was added (indicated by the arrow). The results are the means of experiments conducted with two separate cell preparations. Glycogen phosphorylase *a* activity was measured as described in Materials and Methods.

The concentrations of verapamil and nifedipine which inhibited vasopressin-stimulated Ca^{2+} inflow are considerably higher than those which inhibit potential-operated Ca^{2+} channels present in myocardial muscle cells and in other cell types [27, 31–34] and present results. This comparison indicates that it is unlikely that Ca^{2+} channels with the characteristics of potential-operated Ca^{2+} channels are present in the liver cell plasma membrane. This conclusion is also consistent with the results obtained in the presence of high K^+ buffer. In contrast to increases of 5- to 10-fold in the initial rate of $^{45}Ca^{2+}$ exchange induced by high K^+ buffer in smooth muscle [26] and pituitary cells [27], an increase of only 30% was observed in hepatocytes. Moreover, the concentration of verapamil which inhibits Ca^{2+} exchange in high K^+ buffer in hepatocytes was more than one order of magnitude greater than that which inhibits the beating of isolated heart cells and $^{45}Ca^{2+}$ uptake through potential-operated Ca^{2+} channels in smooth muscle [26] and pituitary cells [27].

Verapamil did not inhibit the initial increase in glycogen phosphorylase induced by vasopressin. Other studies have shown that this increase is primarily due to the release of Ca^{2+} from intracellular stores [10, 35] catalysed by inositol 1,4,5-trisphosphate [10, 36]. Thus it is concluded that it is unlikely that verapamil, at the concentrations employed, inhibits the combination of vasopressin with its receptor or the formation of inositol triphosphate. However, at least one additional effect of verapamil, other than inhibition of plasma membrane Ca^{2+} inflow, was identified in the present study. The transient increase in glycogen phosphorylase observed following the addition of verapamil to hepatocytes does not involve significant alterations in Ca^{2+} transport (as judged by the absence of changes in the intracellular free Ca^{2+} concentration). The mechanism may involve an increase in cyclic AMP concentration (reviewed in ref. 18) and/or direct activation of glycogen phosphorylase.

The observation that verapamil and nifedipine inhibit vasopressin-induced plasma membrane Ca^{2+}

Table 2. Comparison of the properties of liver cell plasma membrane Ca^{2+} inflow transporters with those of potential-operated Ca^{2+} channels in excitable cells

Potential-operated Ca^{2+} channels	Ca^{2+} inflow transporters in hepatocyte plasma membrane
Opened by membrane depolarisation [15]; Ca^{2+} inflow stimulated by hormones and neurotransmitters [15]	Catalyse significant Ca^{2+} inflow in basal state; Ca^{2+} inflow stimulated by hormones [5–12]
Estimated flux of about 10^{-14} mol·cm^{-2}·sec^{-1} [13]	Estimated flux of about 10^{-12} mol·cm^{-2}·sec^{-1} [7]
Inhibited by La^{3+} [42]	Transporters operating in basal state stimulated by La^{3+} [43]
Inhibited by low concentrations Ca^{2+} antagonists [18–21]	Not affected by low concentrations Ca^{2+} antagonists [9, 23, and present results]

inflow with little effect on Ca^{2+} inflow in the basal state indicates that the hormone promotes the activation of Ca^{2+} transporters which differ in nature from those present in the unstimulated cell. Ca^{2+} transporters of this type have been described in pancreatic beta cells [37, 38], lymphocytes [39] and smooth muscle cells [40, 41]. Based on dose–response data for verapamil and nifedipine it is concluded that neither of these channels posess the characteristics of potential-operated Ca^{2+} channels present in excitable cells (Table 2).

Acknowledgements—The provision of verapamil and D600 by the Knoll AG and of compound CGP 28392 by Ciba-Geigy Ltd., Basel, Switzerland, is gratefully acknowledged. The measurement of intracellular free Ca^{2+} concentrations in isolated myocardial muscle cells was performed by Mr James Aspinall. Skilled technical assistance was provided by Ms J. Gunter. This work was supported by a grant from the National Health and Medical Research Council of Australia.

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