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4β-Phorbol 12-myristate 13-acetate attenuates the glucagon-induced increase in cytoplasmic free Ca^{2+} concentration in isolated rat hepatocytes

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1. Hepatocytes were isolated from rats and then loaded with the fluorescent Ca^{2+} indicator quin2. Glucagon caused a sustained increase (at least 5 min) in the fluorescence of the quin2-loaded cells; the increase was much greater than that observed with control, non-quin2-loaded, cells. These observations indicate that glucagon caused an increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$). The effects of glucagon were mimicked if forskolin (to activate adenylate cyclase), dibutyryl cyclic AMP or bromo cyclic AMP were added directly to the cells. Thus an increase in cyclic AMP concentration may mediate the effect of glucagon on $[Ca^{2+}]_c$. 2. If 4β-phorbol 12-myristate 13-acetate (PMA; an activator of protein kinase C) was added to the cells before glucagon, the magnitude of the increase in $[Ca^{2+}]_c$ was greatly diminished. If PMA was added after glucagon it caused a lowering of $[Ca^{2+}]_c$. 3. These effects of PMA on the glucagon-induced increase in $[Ca^{2+}]_c$ could not be mimicked if $[Ca^{2+}]_c$ was increased by the Ca^{2+}-ionophore ionomycin. Thus an event involved in the mechanism by which glucagon increases $[Ca^{2+}]_c$ appears to be required for the action of PMA. 4. If $[Ca^{2+}]_c$ was increased by forskolin, dibutyryl cyclic AMP or bromo cyclic AMP, the effect of PMA on $[Ca^{2+}]_c$ was similar to that observed when glucagon was used to elevate $[Ca^{2+}]_c$. When $[Ca^{2+}]_c$ was raised by dibutyryl cyclic AMP the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine did not prevent the subsequent addition of PMA from causing $[Ca^{2+}]_c$ to decrease. These observations suggest that PMA can inhibit the cyclic AMP-induced increase in $[Ca^{2+}]_c$ independently of any changes in cyclic AMP concentration. 5. Glucagon appears to increase $[Ca^{2+}]_c$ by releasing intracellular stores of Ca^{2+} and stimulating net influx of Ca^{2+} into the cell; PMA greatly diminishes both of these effects.

INTRODUCTION

An increase in $[Ca^{2+}]_c$ is believed to be important in mediating the response of many cell types to external stimuli. Of considerable interest are the mechanisms involved in the hormonal regulation of $[Ca^{2+}]_c$ and the consequences of changes in $[Ca^{2+}]_c$ for cell metabolism. Vasopressin causes an increase in $[Ca^{2+}]_c$ in hepatocytes (Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984) and an increase in the concentration of inositol trisphosphate (Thomas *et al.*, 1984). Inositol trisphosphate may act as a messenger to cause a release of Ca^{2+} from non-mitochondrial intracellular stores, thereby contributing to the increase in $[Ca^{2+}]_c$ (see Berridge & Irvine, 1984; Joseph *et al.*, 1984).

Inositol trisphosphate is believed to be derived from a phospholipase-C-type cleavage of phosphatidylinositol 4,5-bisphosphate; this cleavage also generates diacylglycerol, another potential messenger (see Nishizuka, 1984). The mode of action of diacylglycerol may be via its ability to activate protein kinase C (Kishimoto *et al.*, 1980). Protein kinase C appears to be activated in hepatocytes in response to vasopressin (Garrison *et al.*, 1984), but the functional consequences of this increase in activity remain largely enigmatic.

Not all hormones utilize inositol 1,4,5-trisphosphate as a messenger to increase $[Ca^{2+}]_c$. Charest *et al.* (1983) reported that glucagon could increase $[Ca^{2+}]_c$ in isolated hepatocytes. However, glucagon in hepatocytes caused no increase in the concentration of inositol trisphosphate (Charest *et al.*, 1985). Thus it appears that glucagon increases $[Ca^{2+}]_c$ in hepatocytes by an inositol 1,4,5-trisphosphate-independent mechanism.

The present paper confirms the work of Charest *et al.* (1983) showing that glucagon causes an increase in $[Ca^{2+}]_c$ in hepatocytes. In addition, the mechanisms involved in this increase were investigated. Novel data are also presented which indicate that the activation of protein kinase C attenuates the glucagon-induced increase in $[Ca^{2+}]_c$; the possible mechanisms underlying this phenomenon were also examined. A preliminary report of this work appeared at the 30th Annual Meeting of the U.S. Biophysical Society, San Francisco (Staddon & Hansford, 1986).

MATERIALS AND METHODS

Isolation of hepatocytes

Hepatocytes were isolated from fed 150–300 g male Sprague–Dawley rats; a modification (Krebs *et al.*, 1974) of the Berry & Friend (1969) method was used.

Abbreviations used: Br-cyclic AMP, 8-bromo-cyclic AMP; bt_2-cyclic AMP, $N^6,O^{2'}$-dibutyryl cyclic AMP; $[Ca^{2+}]_c$, cytoplasmic free Ca^{2+} concentration; DTPA, diethylenetriaminepenta-acetic acid; PMA, 4 β-phorbol 12-myristate 13-acetate; quin2/AM, the tetra-acetoxymethyl ester of quin2.

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$[Ca^{2+}]_c$ measurements

The fluorescent dye quin2 was used to measure $[Ca^{2+}]_c$ (Tsien *et al.*, 1982). Quin2 was loaded into the cells via the membrane-permeant quin2/AM (see Tsien *et al.*, 1982). Loading was batchwise in order to generate several samples of cells containing approximately the same amount of quin2.

Isolated hepatocytes were suspended in Krebs & Henseleit (1932) bicarbonate-buffered saline, pH 7.4, containing 0.5% (w/v) exhaustively dialysed bovine serum albumin (fraction V). The final $CaCl_2$ concentration was 2.5 mM. The cells were preincubated for 10 min at 37 °C under a gas phase of CO_2/O_2 (1:19). The cell concentration was about 2.5 mg of cell protein/ml, as determined by a biuret method (Gornall *et al.*, 1949). After the preincubation period, quin2/AM was added to a final concentration of 100 μM from a stock solution (50 mM) in dimethyl sulphoxide. Control cells received the solvent only. The cells were incubated for a further 15 min. They were then centrifuged at 50 *g* for 2 min. The cell pellet was suspended to about 10 mg of cell protein/ml in saline (Krebs & Henseleit, 1932) containing 0.5% albumin. The cells were stored in 1 ml sealed batches on ice and allowed to sediment under gravity.

The regeneration of quin2 from quin2/AM during the loading protocol was assessed. The fluorescence of quin2-loaded cells lysed by Triton X-100 was measured; fluorescence was then quenched by adding Mn^{2+} (Hesketh *et al.*, 1983) at a subsaturating concentration. The quenching of fluorescence found to occur by Mn^{2+} was to the same extent as that observed with standard quin2; as such this indicates full regeneration of the dye. The intracellular concentration of quin2 was estimated to be 1 mM, by comparison of the fluorescence of cell extracts with that of standard quin2 and given a cell volume of 2 μl/mg of cell protein (Krebs *et al.*, 1974).

Fluorescence measurements

Fluorescence was measured with a Farrand Ratio Fluorimeter-2 (Farrand Optical Co., New York, NY, U.S.A.) in single-beam mode. The excitation wavelength of 333 nm was selected by an interference filter (Farrand). The emitted light was measured at wavelengths greater than 480 nm by using filter no. 3–71 from Farrand. The fluorimeter was fitted with a cuvette chamber thermostatically controlled at 37 °C; the cell suspension was maintained by magnetic stirring. Before fluorescence measurements the cell supernatant was removed; the cell pellet was then resuspended in 2 ml of Krebs–Henseleit saline at 37 °C containing 0.2% albumin and, unless indicated otherwise, 2.5 mM-$CaCl_2$. The cells were preincubated in the cuvette for 5–10 min and were continuously gassed with CO_2/O_2 (1:19). Fluorescence was then recorded. Conversion of fluorescence into values for $[Ca^{2+}]_c$ is described in the Results and discussion section.

Materials

Collagenase (type I) was from Cooper. Quin2/AM and ionomycin were from Calbiochem. Albumin, [Arg^8]vasopressin, Br-cyclic AMP, bt_2-cyclic AMP, DTPA, glucagon, isobutylmethylxanthine and PMA were from Sigma Chemical Co. Quin2 was from Molecular Probes. All other chemicals were of the highest grade commercially available. Forskolin was given by Dr. Heinz Metzger (Hoechst, Frankfurt, Germany).

Presentation of results

The fluorescence traces presented are representative of results obtained using several cell preparations. Where a statistical treatment of results has been adopted, the values are means ± S.E.M. for data obtained from the numbers of preparations shown in parentheses. Statistical significance of results was assessed by Student's *t* test.

RESULTS AND DISCUSSION

Effects of glucagon, forskolin and bt_2-cyclic AMP on $[Ca^{2+}]_c$

The Ca^{2+} ionophore ionomycin at a concentration of 10 μM caused a maximal increase in the fluorescence of quin2-loaded hepatocytes (Fig. 1*a*). With the corresponding control (non-quin2-loaded) cells ionomycin caused an increase in fluorescence that was less than 5% of that of the quin2-loaded cells (see Fig. 2). Glucagon at 10 nM, a saturating concentration, also caused an increase in fluorescence of quin2-loaded cells (Fig. 1*b*); this was about 10 times that observed with control cells

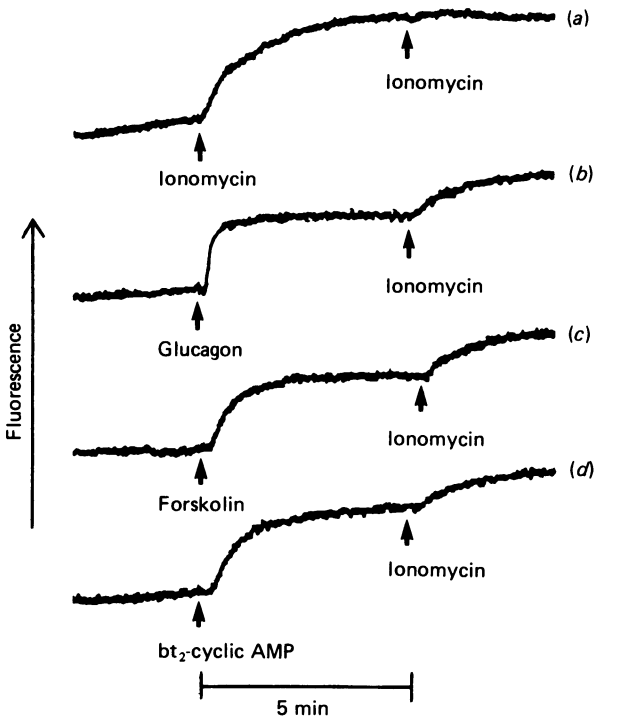

Fig. 1. Effects of ionomycin, glucagon, forskolin and bt_2-cyclic AMP on the fluorescence of quin2-loaded hepatocytes

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. The extracellular Ca^{2+} concentration was 2.5 mM. The final concentrations of compounds added were as follows: 10 μM-ionomycin; 10 nM-glucagon; 10 μM-forskolin; 25 μM-bt_2-cyclic AMP. All compounds were added at maximally effective concentrations. Fluoresence is in arbitrary units. Traces (*a*)–(*d*) were obtained from the same batch of quin2-loaded cells.

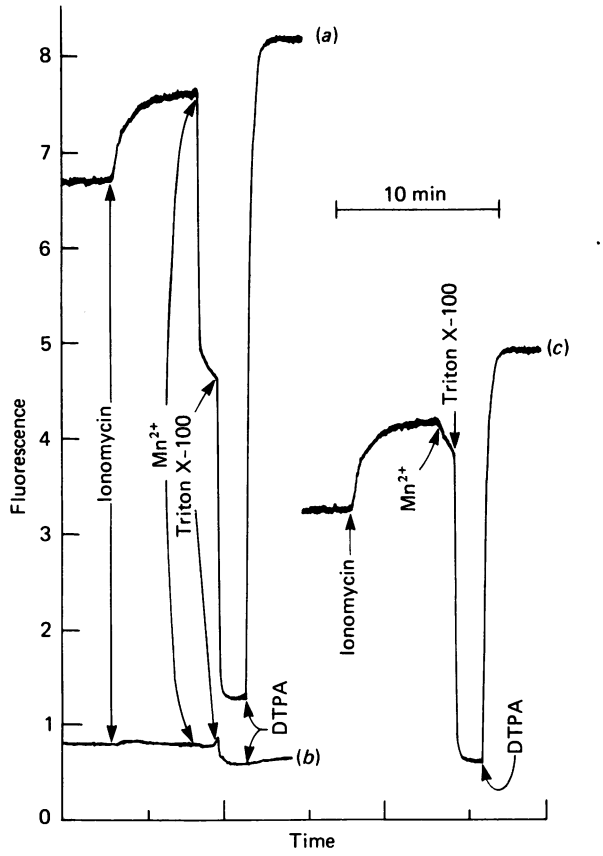

Fig. 2. Calculation of $[Ca^{2+}]_c$ in quin2-loaded hepatocytes

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. Traces (*a*) and (*b*) were obtained, respectively, from quin2-loaded hepatocytes and the corresponding non-quin2-loaded cells. The final $CaCl_2$ concentration was 2.5 mM. Trace (*c*) represents the difference in fluorescence between (*a*) and (*b*) after subtraction of the original decrease observed with addition of Mn^{2+} in (*a*). The final concentrations of compounds added were as follows: 10 μM-ionomycin; 0.1 mM-$MnCl_2$; 0.1% (w/v) Triton X-100; 0.5 mM-DTPA/Ca^{2+} (pH 7.4).

(results not shown). The fluorescence response of the quin2-loaded cells to glucagon indicates an increase in $[Ca^{2+}]_c$, confirming the report by Charest *et al.* (1983). Sistare *et al.* (1985) also made similar observations and established a K_a for glucagon of about 0.3 nM. In the present work, the increase in $[Ca^{2+}]_c$ was sustained for the 5 min interval investigated. The additional increase in fluorescence observed when ionomycin was added after glucagon indicates that the quin2 was not saturated with Ca^{2+} (Fig. 1*b*).

It is well documented that glucagon uses cyclic AMP as a second messenger in liver. Forskolin directly activates adenylate cyclase independently of occupancy of the glucagon receptor (Seamon & Daly, 1981); bt_2-cyclic AMP and Br-cyclic AMP may be used to mimic the action of cyclic AMP. Forskolin (Fig. 1*c*), bt_2-cycylic AMP (Fig. 1*d*) and Br-cyclic AMP (50 μM; results not shown) all caused an increase in $[Ca^{2+}]_c$ that was similar to that observed with glucagon. These observations indicate that cyclic AMP may mediate the increase in $[Ca^{2+}]_c$ seen in response to glucagon. The efficacy of forskolin, bt_2-cyclic AMP and Br-cyclic AMP in raising $[Ca^{2+}]_c$ also makes it unlikely that activation of a receptor-operated Ca^{2+} channel forms part of the mechanism by which glucagon increases $[Ca^{2+}]_c$. Bt_2-cyclic GMP (25 μM) had no effect on $[Ca^{2+}]_c$ (results not shown), which suggests that the effect of bt_2-cyclic AMP on $[Ca^{2+}]_c$ was not due to butyrate formation.

Quantification of the glucagon-induced increase in $[Ca^{2+}]_c$

The fluorescence of quin2-loaded cells is converted into values for $[Ca^{2+}]_c$ by reference to the fluorescence of Ca^{2+}-saturated ($F_{max.}$) and Ca^{2+}-free ($F_{min.}$) cytoplasmic quin2 (Tsien *et al.*, 1982). Fig. 2(*a*) shows the protocol used here to determine $F_{max.}$ and $F_{min.}$. Ionomycin at 10 μM gave $F_{max.}$ (Fig. 2*a*); this concentration was maximally effective (Fig. 1*a*). The subsequent addition of Mn^{2+} caused an instantaneous (< 10 s) quenching (Fig. 2*a*), thus estimating the fluorescence contribution of extracellular quin2. The subsequent slow decline in fluorescence presumably represents permeation of Mn^{2+} into the cell and quenching of intracellular quin2 fluorescence; this was accelerated by lysing the cells with Triton X-100. Triton X-100 also gave a significant decrease in control cell fluorescence (Fig. 2*b*).

When the transition-metal-ion chelator DTPA was added after Triton X-100, an unexpected increase in fluorescence over that obtained after ionomycin was observed (Figs. 2*a* and 2*c*). As ionomycin was added at a maximally effective concentration (Fig. 1*a*), it is unlikely that the cytoplasmic quin2 was not saturated with Ca^{2+}. Additionally, when 15 μg of saponin/mg dry wt. of cells was used to permeabilize the cells maximally (see Joseph *et al.*, 1984), the fluorescence response was similar in magnitude to that observed with ionomycin alone. Digitonin (4 μM) mimicked the action of saponin.

A possible explanation for the described fluorescence enhancement is that, by some mechanism, the fluorescence of intracellular quin2 was quenched; i.e. Triton X-100 relieves quenching by disruption of the cells. It is unlikely that quenching was due to transition-metal ions, as TPEN (25 μM), a membrane-permeant transition-metal-ion chelator (Arslan *et al.*, 1985), had no effect on the fluorescence of quin2-loaded hepatocytes (results not shown). Other possible causes of quenching that cannot be excluded are binding of the dye to cell structures susceptible to disruption by Triton X-100, absorbance of excitation light and/or emitted light. Compartmentation of quin2 is another possibility.

If the fluorescence of intracellular quin2 was quenched, $[Ca^{2+}]_c$ may be estimated as follows. Comparison of the total fluorescence of intracellular dye, i.e. the change in fluorescence after Mn^{2+} and Triton X-100 addition in Fig. 2(*c*), with the total fluorescence of dye released by cell solubilization, i.e. the change in fluorescence after DTPA addition in Fig. 2(*c*), gives an estimate of the degree of quenching. In Fig. 2(*c*) the fluorescence of intracellular quin2 was 82% that of dye released from the cell. For dye released from the cell $F_{min.}$ is calculated by considering the quenching of quin2 itself by Mn^{2+} (see Hesketh *et al.*, 1983). $F_{min.}$ for intracellular dye is then estimated by applying the correction factor (i.e. ×0.82). With $F_{max.}$ taken as that after ionomycin addition, basal $[Ca^{2+}]_c$ is calculated to be 292 ± 10 nM (6); glucagon caused $[Ca^{2+}]_c$ to increase to 1040 ± 151 nM (6; $P < 0.01$). If however, $F_{max.}$ is taken as that obtained after addition

of Triton X-100 plus DTPA, then basal $[Ca^{2+}]_c$ is estimated to be 159 ± 13 nM (6). Binet *et al.* (1985) noted discrepancies for published values of the vasopressin-induced increase in $[Ca^{2+}]_c$ in hepatocytes. These discrepant values may well be due to differences in calibration protocols. Values for $[Ca^{2+}]_c$ obtained by using detergent to estimate $F_{max.}$ will be lower than when ionomycin or digitonin (4 μM) is used in the calibration.

Sources of Ca^{2+} for the glucagon-induced increase in $[Ca^{2+}]_c$

Many hormones that cause an increase in $[Ca^{2+}]_c$ may do so in part by causing the release of Ca^{2+} from intracellular stores (see Berridge & Irvine, 1984). Even when the extracellular $[Ca^{2+}]$ was greatly decreased to about 0.2 μM, glucagon still caused an increase in $[Ca^{2+}]_c$ (Fig. 3*a*); presumably this increase reflects the release of Ca^{2+} from an intracellular store (see also Sistare *et al.*, 1985). As bt_2-cyclic AMP had the same effect as glucagon on $[Ca^{2+}]_c$ (results not shown), this indicates that cyclic AMP, directly or indirectly, mediated the release of the intracellular pool of Ca^{2+}.

In the absence of extracellular Ca^{2+}, the increase in $[Ca^{2+}]_c$ in response to glucagon showed a slow reversal (Fig. 3*a*). However, the response in normal extracellular Ca^{2+} was fully maintained (Fig. 1*b*), suggesting that net influx of Ca^{2+} across the plasma membrane may be required for a sustained increase in $[Ca^{2+}]_c$. Indeed, Mauger *et al.* (1985), in a study using $^{45}Ca^{2+}$, have demonstrated that glucagon stimulates Ca^{2+} influx into hepatocytes.

In the absence of extracellular Ca^{2+}, ionomycin also caused a transient increase in $[Ca^{2+}]_c$ (Fig. 3*b*); a qualitatively similar response to vasopressin is shown in Fig. 3(*c*) (see also Berthon *et al.*, 1984; Cooper *et al.*, 1985). The traces in Figs. 3(*a*)–(*c*) indicate that glucagon, vasopressin and ionomycin all release Ca^{2+} from intracellular stores. In addition, the small magnitude of the increase in fluorescence observed when vasopressin was added after glucagon (Fig. 3*a*) indicates that, at least in the absence of extracellular Ca^{2+}, Ca^{2+} was released from a common intracellular store by the different hormones: reversing the order of hormone additions leads to the same conclusion (Fig. 3*c*). The lack of effect of ionomycin when added after both hormones in either sequence (Figs. 3*a* and 3*c*) indicates that one hormone did not prevent the effect of the other. This latter observation also indicates that Ca^{2+} leaves the cell after its release from the intracellular store. Indeed, measuring Ca^{2+} efflux with Arsenazo III has shown that this does in fact occur (results not shown). In the absence of extracellular Ca^{2+}, as expected, no increase in $[Ca^{2+}]_c$ was observed when glucagon and vasopressin were added after ionomycin (Fig. 3*b*).

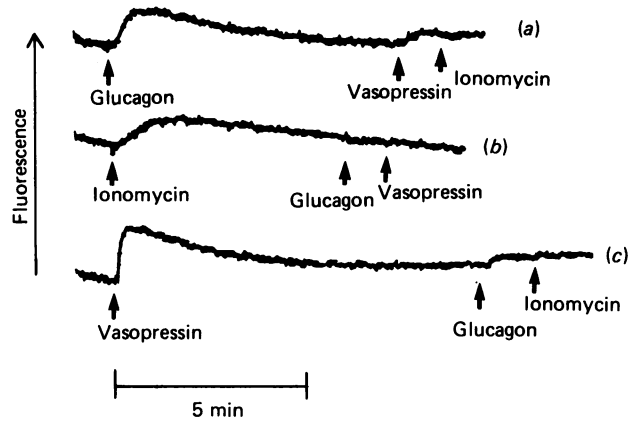

Fig. 3. Effects of glucagon, ionomycin and vasopressin on the fluorescence of quin2-loaded hepatocytes in the absence of extracellular Ca^{2+}

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. The cells were preincubated in the cuvette in the presence of a lower Ca^{2+} concentration, 0.5 mM; to chelate Ca^{2+}, EGTA (pH 7.4) was added (not shown) to a final concentration of 0.75 mM about 100 s before the first addition in each trace. The addition of EGTA resulted in a small instantaneous decrease in fluorescence (attributed to extracellular quin2). The final concentrations of compounds added were as follows: 10 nM-glucagon; 10 μM-ionomycin; 25 nM-vasopressin. Fluorescence is in arbitrary units. Traces (*a*)–(*c*) were obtained from the same batch of quin2-loaded cells.

Effects of PMA on the glucagon-induced increase in $[Ca^{2+}]_c$

When $[Ca^{2+}]_c$ in hepatocytes was increased by glucagon, the subsequent addition of the phorbol ester PMA resulted in a decrease in $[Ca^{2+}]_c$ (Fig. 4*a*). When PMA was added alone to hepatocytes, it had no effect on $[Ca^{2+}]_c$. However, the magnitude of the increase in $[Ca^{2+}]_c$ caused by a subsequent addition of glucagon was greatly decreased (Fig. 4*b*). As measured by the calibration protocol described previously, $[Ca^{2+}]_c$ increased to 481 ± 45 nM ($n = 6$; $P < 0.01$ compared with control and glucagon-treated cells).

The dose–response curve for the effect of PMA on the glucagon-induced increase in $[Ca^{2+}]_c$ is shown in Fig. 5. PMA at 2.5 nM, was effective and concentrations greater than 10 nM had maximum influence; these values are reminiscent of the concentrations required to activate protein kinase C, where the K_a was about 2.5 nM (Castagna *et al.*, 1982). The effects of PMA on $[Ca^{2+}]_c$ may therefore involve an activation of protein kinase C. Consistent with this proposal is the inability of 500 nM-4β-phorbol either to activate protein kinase C (Castagna *et al.*, 1982) or to attenuate the increase in $[Ca^{2+}]_c$ caused by glucagon (results not shown). PMA apparently activates protein kinase C by mimicking the physiological activator diacylglycerol (Castagna *et al.*, 1982).

If sub-saturating amounts of ionomycin were used to elevate $[Ca^{2+}]_c$ to values comparable with those achieved by glucagon addition, the subsequent addition of PMA had no effect on $[Ca^{2+}]_c$ (Fig. 4*d*). Equally, the addition of PMA before ionomycin had no influence on the ability of the ionophore to increase $[Ca^{2+}]_c$ (results not shown). Thus it appears that PMA affects the mechanism by which glucagon raises $[Ca^{2+}]_c$, rather than simply affecting the response of the cell to an increase in $[Ca^{2+}]_c$.

As discussed, cyclic AMP may mediate the action of glucagon in increasing $[Ca^{2+}]_c$. PMA partly inhibited the glucagon-induced increase in cyclic AMP concentration in hepatocytes (Heyworth *et al.*, 1984; García-Sáinz *et al.*, 1985); this partial inhibition could, in theory, explain the attenuation by PMA of the glucagon-induced increase in $[Ca^{2+}]_c$ (Figs. 4*a* and 4*b*). Although not

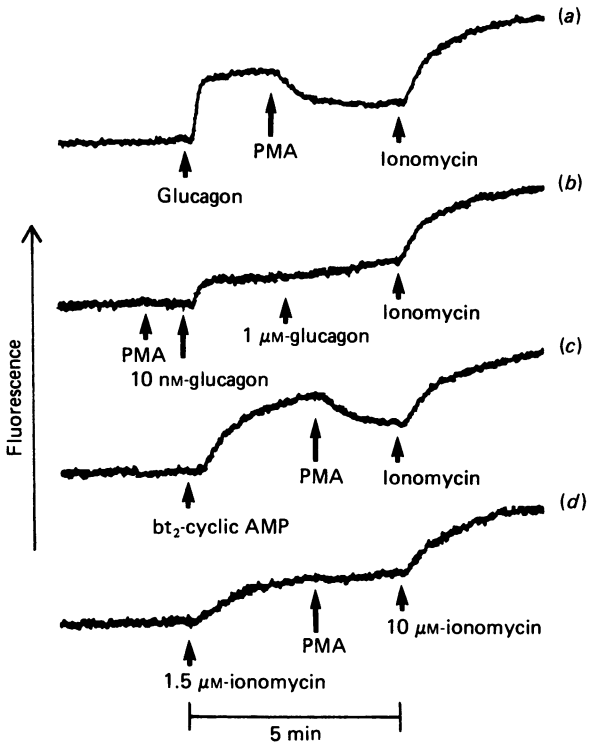

Fig. 4. Effects of PMA on the glucagon-induced increase in the fluorescence of quin2-loaded hepatocytes

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. The extracellular Ca^{2+} concentration was 2.5 mM. The final concentrations of compounds added, unless indicated otherwise, were: 500 nM-PMA; 10 nM-glucagon; 10 μM-ionomycin; 25 μM-bt_2-cyclic AMP. Fluorescence is in arbitrary units. Traces (*a*)–(*d*) were obtained from the same batch of quin2-loaded cells.

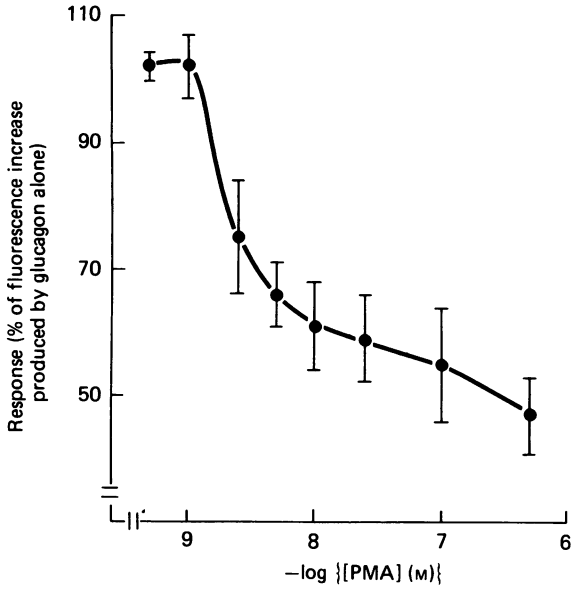

Fig. 5. Concentration-dependence of the effect of PMA on the glucagon-induced increase in the fluorescence of quin2-loaded hepatocytes

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements made as described in the Materials and methods section. The extracellular Ca^{2+} concentration was 2.5 mM. The cells were preincubated with the required concentration of PMA for 3 min. Glucagon was then added to a final concentration of 10 nM. The values shown were obtained from three independent cell preparations, except the value for 500 nM-PMA ($n = 7$).

excluding a role for this inhibition of cyclic AMP production, there are several lines of evidence indicating that PMA is capable of acting independently of changes in cyclic AMP concentration. (1) Increasing the concentration of glucagon from 10 nM to 1 μM in the presence of PMA resulted in an increase in cyclic AMP concentration (García-Sáinz *et al.*, 1985), but the inhibitory effect of PMA on $[Ca^{2+}]_c$ was not surmounted (Fig. 4*b*). (2) Apparently PMA inhibits the glucagon-stimulated adenylate cyclase (Heyworth *et al.*, 1984). However, when $[Ca^{2+}]_c$ was increased by bt_2-cyclic AMP, a subsequent addition of PMA could decrease $[Ca^{2+}]_c$ (Fig. 4*c*), i.e. the decrease was independent of any possible effects on cyclic AMP generation. (3) The effect of PMA is also unlikely to be at the level of cyclic AMP phosphodiesterase, as PMA still attenuated the bt_2-cyclic AMP-induced increase in $[Ca^{2+}]_c$ even in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.5 mM; results not shown). Additionally, PMA did not affect the activity of phosphodiesterase located in the plasma-membrane or 'dense vesicle' fractions of hepatocytes (see Heyworth *et al.*, 1985). Also, similar effects of PMA on $[Ca^{2+}]_c$ (results not shown) were obtained if 50 μM-Br-cyclic AMP was used instead of bt_2-cyclic AMP; Br-cyclic AMP is apparently a poor substrate for phosphodiesterases (Miller *et al.*, 1975). In conclusion, PMA appears to be capable of exerting its influence on the glucagon-induced increase in $[Ca^{2+}]_c$ by a mechanism independent of changes in cyclic AMP concentration.

Influence of PMA on the ability of glucagon to increase $[Ca^{2+}]_c$ in the absence of extracellular Ca^{2+}

Part of the mechanism by which glucagon increases $[Ca^{2+}]_c$ in hepatocytes involves release of Ca^{2+} from an intracellular store. In the absence of extracellular Ca^{2+}, glucagon caused a transient increase in $[Ca^{2+}]_c$ (Figs. 3*a* and 6*a*). When PMA was added before glucagon the effect of the hormone was greatly decreased (Fig. 6*b*), probably because the intracellular store of Ca^{2+} was not released, as the subsequent addition of ionomycin could cause an increase in $[Ca^{2+}]_c$ (Fig. 6*b*). Again, PMA appears to be capable of acting independently of cyclic AMP, because it prevented bt_2-cyclic AMP from releasing the intracellular pool of Ca^{2+} (results not shown).

When PMA was added soon after glucagon, $[Ca^{2+}]_c$ returned to prestimulated values faster than with glucagon alone (cf. Figs. 6*c* and 6*a*). The time required for $[Ca^{2+}]_c$ to return to prestimulated values when PMA was added after glucagon was 1.58 ± 0.15 min (7); the corresponding time in the absence of PMA was 4.45 ± 0.24 min (7) ($P < 0.001$). The subsequent addition of ionomycin caused an increase in $[Ca^{2+}]_c$ (Fig. 6*c*) and, as such, indicates that the addition of PMA had caused a re-uptake of Ca^{2+} into an intracellular store.

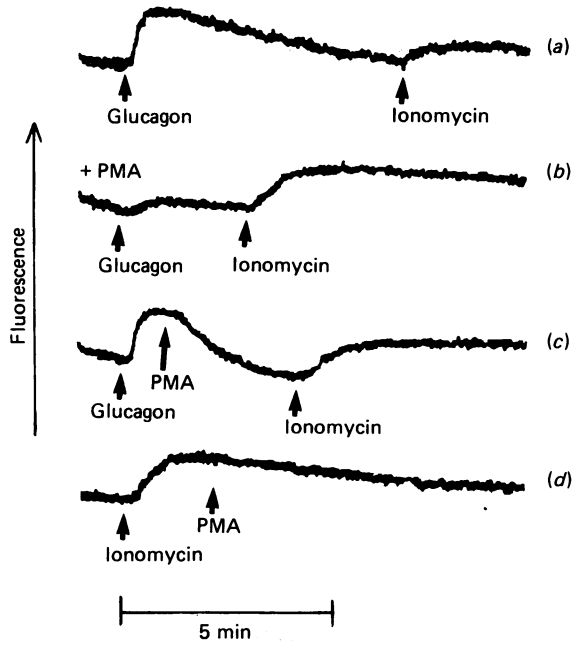

Fig. 6. Effects of PMA on the glucagon-induced increase in the fluorescence of quin2-loaded hepatocytes in the absence of extracellular Ca^{2+}

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. The cells were preincubated in the presence of 0.5 mM-Ca^{2+}, and then extracellular Ca^{2+} was chelated by the addition of EGTA as described in the legend of Fig. 3. The final concentrations of compounds added were: 10 nM-glucagon; 10 μM-ionomycin; 500 nM-PMA. Fluorescence is in arbitrary units. Traces (*a*)–(*d*) were obtained from the same batch of quin2-loaded cells.

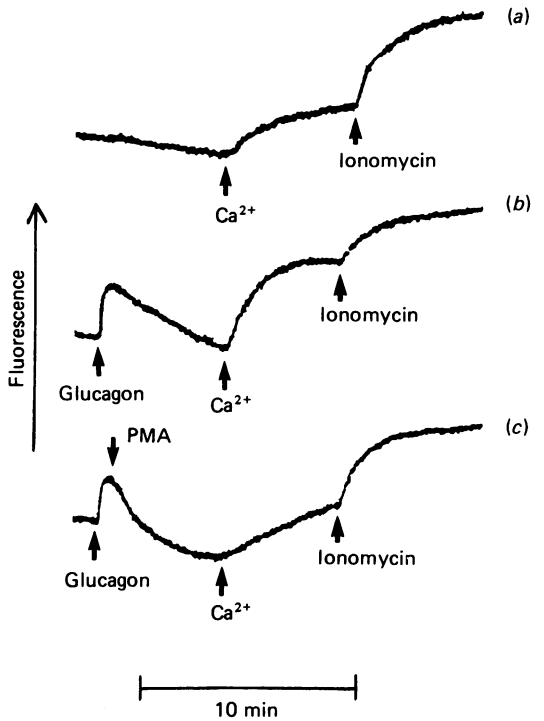

Fig. 7. Effect of glucagon and PMA on the fluorescence of quin2-loaded hepatocytes when Ca^{2+} was restored to cells previously incubated in the absence of extracellular Ca^{2+}

Hepatocytes were isolated and loaded with quin2, and fluorescence measurements were made as described in the Materials and methods section. The cells were preincubated in the presence of 0.5 mM-Ca^{2+}, then Ca^{2+} was chelated as described in the legend to Fig. 3. Ca^{2+} was added back to a final concentration of 2.75 mM. When this addition was made, an instantaneous increase in fluorescence was observed; this was attributed to the presence of extracellular quin2, and for clarity of presentation has been omitted. The final concentrations of compounds added were: 10 nM-glucagon; 500 nM-PMA; 10 μM-ionomycin; 2.75 mM-$CaCl_2$. Fluorescence is an arbitrary units. Traces (*a*)–(*c*) were obtained from the same batch of quin2-loaded cells.

When intracellular Ca^{2+} was released by ionomycin, the subsequent addition of PMA did not accelerate the rate of return of $[Ca^{2+}]_c$ (Fig. 6*d*), as indicated by control experiments (results not shown). Also, when PMA was added before ionomycin the ability of the ionophore to increase $[Ca^{2+}]_c$ was not affected. These observations suggest that PMA in hepatocytes does not influence the efflux of Ca^{2+} via the plasma membrane, a conclusion also reached by Garrison *et al.* (1984) and Cooper *et al.* (1985).

Influence of PMA on the ability of glucagon to increase net influx of Ca^{2+} into the cell

In the absence of extracellular Ca^{2+}, glucagon caused a transient increase in $[Ca^{2+}]_c$ (Fig. 7*b*), as has been shown above (Figs. 3*a* and 6*a*). When Ca^{2+} was added back to cells incubated in the absence of extracellular Ca^{2+}, then the increase in $[Ca^{2+}]_c$ was greater in glucagon-treated cells (Fig. 7*b*) than in control cells (Fig. 7*a*), indicating that glucagon stimulated net influx of Ca^{2+} into the cell.

If PMA was added soon after glucagon in the absence of extracellular Ca^{2+}, the rate of decrease in $[Ca^{2+}]_c$ was enhanced (Fig. 7*c*), as described previously. However, when Ca^{2+} was restored to the medium, the increase in fluorescence was comparable with that observed with cells not treated with glucagon (Fig. 7*a*). Although glucagon increases net Ca^{2+} influx into hepatocytes, it appears that PMA can abolish this stimulation.

General discussion

Although it has been known for more than 20 years that glucagon increases the concentration of cyclic AMP in liver, it has only more recently been established that the hormone also increases $[Ca^{2+}]_c$ (Charest *et al.*, 1983; Sistare *et al.*, 1985; the present work). This is a rather intriguing observation, because most changes in cytoplasmic enzyme activity caused by glucagon can readily be accounted for by an activation of cyclic AMP-dependent protein kinase. However, glucagon can influence mitochondrial metabolism in hepatocytes; for example, it activates the intramitochondrial Ca^{2+}-sensitive enzymes pyruvate dehydrogenase and oxoglutarate dehydrogenase (see McCormack, 1985). If an increase in $[Ca^{2+}]_c$ results in an increase in the intramitochondrial free Ca^{2+} concentration, then the glucagon-induced increase in $[Ca^{2+}]_c$ may account for the activation of the aforementioned enzymes (see McCormack, 1985).

The molecular mechanisms involved in Ca^{2+} mobilization by cyclic AMP, and its antagonism by protein kinase C, remain to be established. It seems likely, however, that the major pool of intracellular Ca^{2+} in liver that can be mobilized by hormones is localized in the endoplasmic reticulum (see Kleineke & Söling, 1985; Somlyo *et al.*, 1985), and that this is the 'store' discussed in the present paper. Also, the physiological significance of a mechanism whereby the activation of protein kinase C antagonizes the mobilization of Ca^{2+} by glucagon is unclear. The present work does, however, demonstrate a potential interaction between the cylic AMP and diacylglycerol signal transduction mechanisms.

In summary, the increase in $[Ca^{2+}]_c$ in response to glucagon in hepatocytes may be a consequence of an increase in cyclic AMP concentration. PMA attenuates the increase in $[Ca^{2+}]_c$ caused by glucagon; the mechanism is, at least in part, independent of changes in cyclic AMP concentration. The action of PMA may be mediated by protein kinase C, and counteracts the ability of glucagon to stimulate both release of Ca^{2+} from an intracellular store and net Ca^{2+} influx into the cell.

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