The stimulation by sodium fluoride of plasma-membrane Ca^{2+} inflow in isolated hepatocytes

Evidence that a GTP-binding regulatory protein is involved in the hormonal stimulation of Ca^{2+} inflow

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1. In isolated hepatocytes NaF increased the rate of $^{45}\text{Ca}^{2+}$ exchange, the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (monitored by using quin2), and the activity of glycogen phosphorylase *a* in a Ca^{2+} -dependent manner. 2. In cells previously incubated in the absence of extracellular Ca^{2+} (Ca^{2+}_o), NaF caused a pronounced enhancement in the increases in the activity of glycogen phosphorylase and in $[\text{Ca}^{2+}]_i$ observed when Ca^{2+} was subsequently added. The effect of NaF on glycogen phosphorylase activity was inhibited by verapamil and deferoxamine, and was potentiated by AlCl_3 . 3. The actions of NaF were associated with (a) increases in $[\text{H}^3]$ inositol polyphosphates, which were slower in onset and about half the magnitude of those induced by vasopressin, in hepatocytes labelled with $[\text{H}^3]$ inositol, and (b) enhanced rates of O_2 utilization and decreased concentrations of ATP. The latter effects were not potentiated by AlCl_3 . 4. Preincubation of hepatocytes with vasopressin in the absence of added Ca^{2+}_o for times up to 30 min did not diminish the ability of a subsequent addition of extracellular Ca^{2+} to activate glycogen phosphorylase. 5. 12-*O*-Tetradecanoylphorbol 13-acetate had little effect on $^{45}\text{Ca}^{2+}$ exchange and did not enhance the activation by Ca^{2+}_o of phosphorylase in hepatocytes incubated in the absence of Ca^{2+}_o . 6. On the basis of the observation that AlF_4^- activates GTP-binding regulatory proteins [Sternweiss & Gilman (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4888–4891], it is concluded that the present results provide evidence for the function of a GTP-binding regulatory protein in the mechanism by which hormones stimulate plasma-membrane Ca^{2+} inflow in the liver cell, and indicate that an increase in $[\text{Ca}^{2+}]_i$ and the activation of protein kinase C are not part of this mechanism.

INTRODUCTION

The actions of α_1 -adrenergic agonists, vasopressin and angiotensin II on liver cells are associated with a stimulation of phosphoinositide hydrolysis (Billah & Michell, 1979; Creba *et al.*, 1983) and increases in the intracellular concentrations of inositol polyphosphates (Burgess *et al.*, 1984; Thomas *et al.*, 1984), the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Charest *et al.*, 1983) and diacylglycerol (Hughes *et al.*, 1984; Bocckino *et al.*, 1985). The stimulation of phosphoinositide hydrolysis, which is catalysed by phosphoinositide-specific phospholipase C (reviewed by Berridge, 1984), requires a GTP-binding regulatory protein (Blackmore *et al.*, 1985; Uhing *et al.*, 1986).

The increase in $[\text{Ca}^{2+}]_i$ is caused by increases in both plasma-membrane Ca^{2+} inflow (Barritt *et al.*, 1981) and net Ca^{2+} outflow from the endoplasmic reticulum (Shears & Kirk, 1984) and possibly other intracellular organelles (Blackmore *et al.*, 1979; Reinhart *et al.*, 1982). The increase in Ca^{2+} outflow from the endoplasmic reticulum is induced by inositol 1,4,5-trisphosphate (InsP_3) (Burgess *et al.*, 1984; Joseph *et al.*, 1984). Evidence for the presence of agonist-activated Ca^{2+} -inflow transporters in the liver cell plasma membrane has been obtained (Barritt *et al.*, 1981; Parker *et al.*, 1983; Mauger *et al.*,

1984; Hughes *et al.*, 1986a). However, it is at present unclear whether these Ca^{2+} transporters are the same as (Mauger *et al.*, 1984) or are different from (Hughes *et al.*, 1986a) Ca^{2+} transporters present in the absence of agonists.

The nature of the link between hormone receptors and plasma-membrane Ca^{2+} -inflow transporters is not well understood. It may involve increases in the intracellular concentrations of InsP_3 (Slack *et al.*, 1986; Putney, 1986), inositol 1,3,4,5-tetrakisphosphate (Hansen *et al.*, 1986) or Ca^{2+} (Petersen & Murayama, 1983; Putney, 1986), the activation by diacylglycerol of protein kinase C (Cooper *et al.*, 1984; and cf. Albert & Tashjian, 1985; Harris *et al.*, 1986; Wakade *et al.*, 1986) or the interaction of a GTP-binding regulatory protein with the Ca^{2+} transporters by a process which does not involve protein kinase C (cf. Molski *et al.*, 1984; Pfaffinger *et al.*, 1985). Cyclic AMP is unlikely to be involved (Kirk *et al.*, 1981).

The aim of the present experiments was to investigate the roles of GTP-binding regulatory proteins, inositol polyphosphates, protein kinase C and increases in $[\text{Ca}^{2+}]_i$ in the mechanism by which vasopressin stimulates agonist-activated plasma-membrane Ca^{2+} -inflow transporters in the liver cell. Investigation of the role of GTP-binding regulatory proteins was based on the

Abbreviations used: InsP , *myo*-inositol monophosphate; InsP_2 , *myo*-inositol bisphosphate; InsP_3 , *myo*-inositol trisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $[\text{Ca}^{2+}]_o$, concentration of extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_i$, concentration of free cytosolic Ca^{2+} .

observations that (i) AlF_4^- activates purified GTP-binding regulatory proteins by mimicking the action of GTP on the α subunit (Sternweiss & Gilman, 1982) and (ii) in the liver cell addition of NaF alone, or a combination of NaF and AlCl_3 , appears to mimic the action of agonists in activating the GTP-binding regulatory protein which is proposed to be coupled to phosphoinositide-specific phospholipase C (Blackmore *et al.*, 1985; Blackmore & Exton, 1986). The results presented here provide evidence which indicates that a GTP-binding regulatory protein plays a role in the mechanism by which hormones stimulate plasma-membrane Ca^{2+} inflow. Furthermore, evidence which indicates that subsequent increases in $[\text{Ca}^{2+}]_i$ and the action of protein kinase C are not part of this mechanism has been obtained.

EXPERIMENTAL

The isolation of hepatocytes from fed male rats, incubation of cells and measurement of $^{45}\text{Ca}^{2+}$ exchange were performed as described previously (Barritt *et al.*, 1981; Hughes *et al.*, 1986b). The incubation medium contained 117 mM-NaCl, 4.6 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 , 20 mM-Tes/NaOH, 1.3 mM- CaCl_2 (except where indicated otherwise) and hepatocytes [2×10^6 cells (30 mg wet wt.)/ml]. Glycogen phosphorylase *a* activity was assayed by using $[\text{U}-^{14}\text{C}]$ -glucose 1-phosphate as described by Hutson *et al.* (1976). A unit of enzyme activity is 1 μmol of $[\text{U}-^{14}\text{C}]$ -glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells. The preparation of hepatocytes loaded with quin2, measurement of quin2 fluorescence, and the calculation of $[\text{Ca}^{2+}]_i$ (Hughes *et al.*, 1986b), the measurement of rates of O_2 consumption (Whiting & Barritt, 1982), and measurement of the amounts of ATP and cyclic AMP in HClO_4 extracts of cell incubation mixtures (Parker *et al.*, 1983; Whiting & Barritt, 1982) were conducted as described previously.

Hepatocytes labelled with $[\text{H}]\text{inositol}$ were prepared by the incubation of cells for 60 min in Incubation Medium, which contained 14 mM-glucose, 1.5% (w/v) gelatin and *myo*- $[\text{H}]\text{inositol}$ (13 $\mu\text{Ci}/\text{ml}$). Incubations were conducted in 250 ml glass flasks, shaken at 80 oscillations/min at 37 °C and gassed (1 litre/min) with O_2/CO_2 (19:1). The cells were washed once and resuspended (45 mg wet wt./ml) in Incubation Medium. The incubation of $[\text{H}]\text{inositol}$ -labelled cells (1.5 ml total volume) was conducted at 37 °C in 20 ml sealed plastic pots, shaken at 90 oscillations/min under O_2/CO_2 (19:1). Trichloroacetic acid extracts were prepared and the separation of inositol polyphosphates was conducted as described by Charest *et al.* (1985), except that AG1-X8 resin was used in place of Dowex 1-X8 resin. Control experiments indicated that the two resins gave similar elution profiles. Columns of about 1.5 ml of AG1-X8 resin (formate form) in disposable 5 ml pipette tips plugged with glass wool were eluted sequentially as follows: 2×5 ml of water (inositol), 2×5 ml of 5 mM-sodium tetraborate/60 mM-sodium formate (glycerophosphoinositol), 4×5 ml of 5 mM-sodium tetraborate/180 mM-sodium formate (InsP), 10×5 ml of 0.1 M-formic acid/0.4 M-ammonium formate (InsP_2) and 4×5 ml of 0.1 M-formic acid/1.0 M-ammonium formate (InsP_3). Each fraction (5 ml) was mixed with 10 ml of ACS II scintillation cocktail, and the amount of ^3H was

determined in an Isocap/300 (Searle Analytic) liquid-scintillation counter.

Except where indicated otherwise, the results are means $\pm$ S.E.M. of the numbers of experiments indicated. Degrees of significance were determined by Student's *t* test for paired samples. Values of $P > 0.05$ were considered to be not significant.

$[\text{U}-^{14}\text{C}]$ Glucose 1-phosphate, *myo*- $[\text{H}]\text{inositol}$ and ACS II were purchased from Amersham (Australia), Surrey Hills, N.S.W., Australia, and AG1-X8 (100–200 mesh) resin was from Bio-Rad Laboratories, Richmond, CA, U.S.A. NaF was from Sigma Chemical Co., St. Louis, MO, U.S.A. Verapamil was kindly provided by Knoll A. G., Ludwigshafen, Germany. Deferoxamine (desferrioxamine mesylate) was from Ciba-Geigy Australia, Lane Cove, N.S.W., Australia. All other chemicals were of the highest grade available and were obtained from the sources described previously (Whiting & Barritt, 1982; Hughes *et al.*, 1986b).

RESULTS

Stimulation by NaF of plasma-membrane Ca^{2+} inflow

In the presence of 1.3 mM- Ca^{2+}_o , incubation of hepatocytes with NaF for 10 min before the addition of tracer $^{45}\text{Ca}^{2+}$ caused a stimulation of about 35% in the rate of $^{45}\text{Ca}^{2+}$ exchange (Fig. 1). Decreasing the time of exposure to NaF to 5 min caused an increase of only 10% (results not shown). Exposure of hepatocytes to

Fig. 1. Stimulation by NaF of $^{45}\text{Ca}^{2+}$ exchange

$^{45}\text{Ca}^{2+}$ exchange by isolated hepatocytes was measured in the absence (○) or presence (●) of 18 mM-NaF. When present NaF was added 10 min before tracer $^{45}\text{Ca}^{2+}$. The data are the means $\pm$ S.E.M. for experiments conducted with three preparations of hepatocytes. The degree of significance is * $P < 0.05$.

Fig. 2. Activation by NaF of glycogen phosphorylase

The activity of glycogen phosphorylase *a* was measured at the times shown as described in the Experimental section. (a) Hepatocytes were incubated in the absence (○) or presence (●) of 1.3 mM- CaCl_2 for 30 min before the addition of NaF (indicated by the arrow). Control experiments indicated that addition of 18 mM-NaCl in place of NaF had no effect on glycogen phosphorylase activity (results not shown). (b) Hepatocytes were incubated in the presence of 1.3 mM- CaCl_2 for 20 min before the addition of dimethyl sulphoxide (final concn. 0.5%, v/v) (○) or 200 μM-verapamil (●). At 30 min, 18 mM-NaF (indicated by the arrow) was added. The degree of significance is * $P < 0.01$. The data (a and b) are the means $\pm$ S.E.M. of experiments conducted with three cell preparations.

Fig. 3. Activation by Ca^{2+} of glycogen phosphorylase in hepatocytes previously incubated in the absence of Ca^{2+} but in the presence of NaF

Hepatocytes were incubated in the absence of added Ca^{2+} . Dimethyl sulphoxide (0.5%, v/v) (○) or 200 μM-verapamil (●) was added after 10 min, 16 mM-NaF at 20 min, and 1.3 mM- CaCl_2 (indicated by the arrow) at 30 min. Verapamil was dissolved in dimethyl sulphoxide (final concn. 0.5%). The data are means $\pm$ S.E.M. of experiments conducted with three cell preparations.

18 mM-NaF for times up to 10 min did not alter the viability of the cells, as judged by exclusion of Trypan Blue (results not shown).

As shown by Blackmore *et al.* (1985), NaF increased the activity of glycogen phosphorylase *a* (Fig. 2a). In the absence of added Ca^{2+} , the increase in glycogen phosphorylase was diminished and the rate of the decline in enzyme activity was slightly greater (Fig. 2a). Verapamil substantially inhibited the increase in glycogen phosphorylase *a* activity caused by NaF at 10 min after the addition of NaF (Fig. 2b). No inhibition was observed at earlier times. Inhibition by verapamil was abolished when $[\text{Ca}^{2+}]_o$ was increased to 10 mM. [The values for glycogen phosphorylase activity at 10 min after NaF addition were 20 and 21 units (means of two experiments) in the presence and absence of verapamil respectively.]

It has previously been shown that the addition of 1.3 mM- Ca^{2+}_o to hepatocytes incubated in the absence of added Ca^{2+}_o causes a substantial increase in glycogen phosphorylase *a* activity in the presence, but not in the absence, of vasopressin (Cooper *et al.*, 1985; Hughes *et al.*, 1986b). In cells pre-treated with NaF in the absence of added Ca^{2+}_o , addition of Ca^{2+} increased glycogen phosphorylase *a* activity. This effect was completely inhibited by verapamil (Fig. 3) (cf. Hughes *et al.*, 1986a). The concentration of verapamil required for 50% inhibition was about 50 μM (results not shown).

Fig. 4 shows the effect of pre-treatment of hepatocytes with 5 mM-NaF on the increase in glycogen-phosphorylase *a* activity caused by the subsequent addition of Ca^{2+} . Treatment with NaF for 8 min (Fig. 4b) resulted in a faster rate of increase in glycogen phosphorylase activity than that observed after exposure to NaF for 2 min (Fig. 4a). Pre-treatment of hepatocytes with deferoxamine, a strong chelator of trivalent metal ions (Ackrill & Day, 1984), inhibited, and addition of

Fig. 4. Effects of deferoxamine and AlCl_3 on the ability of NaF to permit Ca^{2+} stimulation of glycogen phosphorylase a

Hepatocytes were incubated in the absence of added Ca^{2+} in the presence or absence of 0.5 mM-deferoxamine. At 14 min, either saline or 15 μM - AlCl_3 was added. At 15 min, 5 mM-NaF was added to all incubations. At 17 (a) or 23 (b) min a sample was removed from each incubation (for measurements of enzyme activity), and 1.3 mM- CaCl_2 was added immediately, as indicated by the arrow, to all incubations (0 s on the graph). During the next 60 s further samples were removed for the measurement of enzyme activity. Key: $\circ$, control; $\bullet$, deferoxamine; $\blacktriangle$, AlCl_3 . The values are means $\pm$ S.E.M. of experiments conducted with four cell preparations or the means of experiments with two cell preparations. In cells which were not exposed to NaF, the increases in glycogen phosphorylase observed at 30 and 60 s after Ca^{2+} addition were about 2 and 4 units respectively (results not shown).

exogenous AlCl_3 potentiated, both the effect of NaF on glycogen phosphorylase activity in the absence of added Ca^{2+} , as indicated by the values at 0 min in Fig. 4 (cf. Blackmore *et al.*, 1985), and the rate of increase in enzyme activity after the addition of Ca^{2+} (Fig. 4). Deferoxamine had no effect on cell viability, as judged by measurement of the exclusion of Trypan Blue (results not shown). In the absence of NaF, AlCl_3 had no effect on glycogen phosphorylase activity (results not shown). Furthermore, incubation of hepatocytes with either deferoxamine or AlCl_3 in the presence of 1.3 mM- Ca^{2+} had no effect on the increase in glycogen phosphorylase activity caused by subsequent addition of vasopressin (results not shown).

The addition of 18 mM-NaF to hepatocytes incubated in the presence of 1.3 mM- Ca^{2+} increased $[\text{Ca}^{2+}]_i$ as monitored by quin2 fluorescence (Fig. 5a). This confirms

Fig. 5. Effects of NaF on the cytoplasmic free Ca^{2+} concentration

Measurement of the fluorescence of hepatocytes loaded with quin2, and calculation of $[\text{Ca}^{2+}]_i$, were performed as described in the Experimental section. Hepatocytes loaded with quin2 were incubated in the presence of 1.3 mM- Ca^{2+}_o (a) or in the absence of added Ca^{2+}_o (b and c), and in the presence of 1% (v/v) dimethyl sulphoxide (b) or 400 μM -verapamil in dimethyl sulphoxide (c). NaF (18 mM) and CaCl_2 (1.3 mM) were added at the times indicated by the arrows. The traces shown are from one of two experiments which gave similar results. The numbers above the traces are values (nm) of $[\text{Ca}^{2+}]_i$ (means of two experiments).

the work of Blackmore *et al.* (1985). A substantial increase in $[\text{Ca}^{2+}]_i$ was observed at 10 min after addition of NaF (Fig. 5a). The time course for the effect of NaF on $[\text{Ca}^{2+}]_i$ was altered in cells incubated in the absence of added Ca^{2+}_o (Fig. 5b). Under these conditions, $[\text{Ca}^{2+}]_i$ had returned to the basal value by 7 min after NaF addition (Fig. 5b). Subsequent addition of 1.3 mM- Ca^{2+}_o increased $[\text{Ca}^{2+}]_i$ (Fig. 5b). This increase was substantially inhibited by verapamil (Fig. 5c).

Blackmore *et al.* (1985) have previously shown that NaF increases the amounts of $[\text{^3H}]$ inositol polyphosphates in isolated hepatocytes. Since changes in inositol polyphosphates induced by NaF were considered essential to interpretation of the present results, the effects of NaF on these metabolites were compared with those of vasopressin under the conditions of the present study. In the absence of Li^+ , the increase in the amount of $[\text{^3H}]\text{InsP}_3$, measured 10 min after addition of NaF, was about half that caused by a maximally active concentration of vasopressin (Table 1). There was little difference in the amounts of $[\text{^3H}]\text{InsP}$ and $[\text{^3H}]\text{InsP}_2$. In the presence of Li^+ , the increase in $[\text{^3H}]\text{InsP}_3$ induced by NaF was about 30% of that caused by vasopressin (Table 1). Moreover, the increases in $[\text{^3H}]\text{InsP}_2$ and $[\text{^3H}]\text{InsP}$ induced by NaF were considerably less than those caused by vasopressin (Table 1).

The effects of the exposure of cells incubated in the absence of Li^+ to 20 mM-NaF for shorter periods of time were also investigated. The amounts of $[\text{^3H}]\text{InsP}$, $[\text{^3H}]\text{InsP}_2$ and $[\text{^3H}]\text{InsP}_3$ (expressed as percentages of controls) were 100%, 91% and 119% respectively at 1 min after NaF addition, and 128%, 153% and 327% respectively at 5 min after the addition of NaF (means of two experiments).

The incubation of hepatocytes with 18 mM-NaF for periods of 1–10 min caused no change in the concentration of cyclic AMP (results not shown). NaF at concentrations of 5 or 18 mM increased the rate of O_2 consumption by 25 and 38% respectively. The effect was evident from 10 s after NaF addition, was maintained for at least 10 min, and was not potentiated by addition of

Table 1. Comparison of the effects of NaF with those of vasopressin on the formation of [³H]inositol polyphosphates in hepatocytes labelled with [³H]inositol incubated in the presence or absence of LiCl

Hepatocytes labelled with [³H]inositol were incubated for 5 min before the addition of vasopressin, NaF, LiCl or 0.9% (w/v) NaCl (control). After a further period of 10 min, trichloroacetic acid extracts were prepared and ³H-labelled inositol polyphosphates separated as described in the Experimental section. For each inositol polyphosphate, the amount of radioactivity present in cells treated with agonist is expressed as a percentage of that present in the corresponding control incubation. The amounts of radioactivity (c.p.m./peak) present in the fractions obtained after chromatography of extracts of control cells were 2288 ± 207 (8), 1159 ± 161 (8) and 62 ± 10 (8) in the absence of Li⁺, and 2564 (2), 1338 (2) and 86 (2) in the presence of Li⁺, for InsP, InsP₂ and InsP₃ respectively. The results shown are means ± S.E.M. of the numbers of experiments shown, or the means of two experiments. The degrees of significance for values greater than the corresponding control are: **P* < 0.01; ***P* < 0.005; ****P* < 0.0005. The degree of significance for a comparison of the amount of radioactivity present in InsP₃ for cells treated with vasopressin and NaF in the absence of Li⁺ is *P* < 0.001 (*t* test for unpaired samples).

Agent under test	Li ⁺ (mM)	Inositol polyphosphate	Radioactivity in inositol polyphosphate (% of control)
Vasopressin (20 nM)	—	InsP	151 ± 7 (6)***
		InsP ₂	293 ± 31 (6)***
		InsP ₃	1103 ± 204 (6)**
NaF (20 mM)	—	InsP	187 ± 13 (7)***
		InsP ₂	231 ± 35 (7)**
		InsP ₃	546 ± 134 (7)*
Vasopressin (20 nM)	10	InsP	296
		InsP ₂	587
		InsP ₃	1340
NaF (20 mM)	10	InsP	173
		InsP ₂	179
		InsP ₃	479

15 μM-AlCl₃. After 10 min incubation with 5 mM- or 18 mM-NaF, cellular ATP concentrations were decreased by 30 and 45% respectively (results not shown). The effect of 5 mM-NaF was not potentiated by AlCl₃ or decreased by deferoxamine.

Roles of [Ca²⁺]_i and protein kinase C

The data in Fig. 6 show that the addition of 1.3 mM-Ca²⁺_o to cells treated as much as 30 min previously with vasopressin caused the same degree of stimulation of glycogen phosphorylase as that observed in cells exposed to vasopressin for only 5 min before the addition of Ca²⁺. TPA stimulated the initial rate of ⁴⁵Ca²⁺ exchange by 10% (*P* > 0.05), compared with a 40% stimulation induced by vasopressin (results not shown). In contrast with vasopressin and NaF, TPA had no effect on the activation by Ca²⁺_o of phosphorylase in cells previously incubated in the absence of added Ca²⁺_o (Fig. 7). The efficacy of TPA was tested by determining the effect of the phorbol ester on adrenaline-stimulated ⁴⁵Ca²⁺ exchange. TPA inhibited the ability of adrenaline to stimulate ⁴⁵Ca²⁺ exchange (results not shown). This result is consistent with the observations that protein kinase C inhibits expression of α₁-adrenergic receptors in hepatocytes (Cooper *et al.*, 1985; Lynch *et al.*, 1985; van de Werve *et al.*, 1985; Corvera *et al.*, 1986).

DISCUSSION

The observation that the addition of Ca²⁺ to hepatocytes previously incubated for 30 min in the absence of Ca²⁺_o and in the presence of vasopressin

activates glycogen phosphorylase indicates that vasopressin enhances plasma-membrane Ca²⁺ inflow at resting values of [Ca²⁺]_i. {It has previously been shown that addition of vasopressin to hepatocytes incubated in the absence of added Ca²⁺_o leads to a transient increase in [Ca²⁺]_i, which is complete within 5 min of addition of the hormone (Binet *et al.*, 1985; Charest *et al.*, 1985; Joseph *et al.*, 1985; Hughes *et al.*, 1986b).} The results of the experiments conducted with TPA indicate that it is unlikely that activation of protein kinase C enhances plasma-membrane Ca²⁺ inflow. Although the possibility that diacylglycerol directly activates Ca²⁺-inflow transporters cannot be excluded, this is considered unlikely. The conclusion that the mechanism by which agonists stimulate plasma-membrane Ca²⁺ inflow does not require an increase in [Ca²⁺]_i above the resting value, or activation of protein kinase C, is consistent with these observations.

The following results indicate that NaF increases plasma-membrane Ca²⁺ inflow in hepatocytes. (1) The stimulation by NaF of the initial rate of ⁴⁵Ca²⁺ exchange; (2) the increase in glycogen phosphorylase activity or quin2 fluorescence induced by NaF in cells incubated at 1.3 mM-Ca²⁺_o at longer as well as shorter times after NaF addition; and (3) the marked enhancement by pre-treatment with NaF of the increase in glycogen phosphorylase activity or quin2 fluorescence which follows the addition of Ca²⁺ to hepatocytes previously incubated in the absence of Ca²⁺. The observation that NaF-stimulated Ca²⁺ inflow is inhibited by verapamil, at a concentration of the drug similar to that which inhibits vasopressin-stimulated Ca²⁺ inflow (Blackmore *et al.*, 1984; Hughes *et al.*, 1986a), provides

Fig. 6. Effect of pre-treatment with vasopressin of hepatocytes incubated in the absence of added Ca^{2+} on the ability of a subsequent addition of Ca^{2+} to activate glycogen phosphorylase

Hepatocytes were incubated in the absence of added Ca^{2+} and in the absence (○) or presence (●, ■, ▲) of vasopressin. The hormone was added at 0 (■), 15 (▲) or 25 min (●). At 30 min, 1.3 mM- $CaCl_2$ was added to all incubations (indicated by the arrow). Samples were taken for the measurement of glycogen phosphorylase activity at the indicated times. The data are means of experiments conducted with two cell preparations.

some evidence that NaF activates the same Ca^{2+} transporters as those activated by hormones.

There are a number of processes, in addition to the activation by AlF_4^- of a GTP-binding regulatory protein (Sternweiss & Gilman, 1982; Blackmore *et al.*, 1985; Guillon *et al.*, 1986; Blackmore & Exton, 1986), which could lead to the stimulation by NaF of plasma-membrane Ca^{2+} inflow and/or increases in $[Ca^{2+}]_i$ and glycogen phosphorylase activity. These include increases in cyclic AMP (Katada *et al.*, 1984), inhibition of phosphoprotein phosphatase(s) specific for the phosphorylated form of glycogen phosphorylase (Khandelwal, 1977), inhibition of Ca^{2+} transporters (cf. Blackmore *et al.*, 1985) and inhibition of other enzymes, including enolase (reviewed by Bücher, 1955). However, the following observations indicate that these are unlikely explanations for the actions of NaF. (1) NaF caused no change in the concentration of cyclic AMP under the conditions of the present experiments (cf. Blackmore *et al.*, 1985). (2) As discussed by Blackmore *et al.* (1985), there is at present no evidence for the

Fig. 7. Effect of pre-treatment with TPA, NaF or vasopressin of hepatocytes incubated in the absence of added Ca^{2+} on the ability of a subsequent addition of Ca^{2+} to activate glycogen phosphorylase

Hepatocytes were incubated in the absence of added Ca^{2+} before the addition of 18 mM-NaF at 20 min (▲), or 10 nM-vasopressin (■), TPA (1 μ g/ml) dissolved in 1% (final concn.) dimethyl sulphoxide (●) or dimethyl sulphoxide (1%) (○) at 25 min. At 30 min, 1.3 mM- $CaCl_2$ (indicated by the arrow) was added to all incubations. Samples were removed for measurement of glycogen phosphorylase activity at the indicated times. The data are means $\pm$ S.E.M. of experiments conducted with three cell preparations.

inhibition by F^- of cellular Ca^{2+} transporters. (3) $AlCl_3$ potentiated the ability of NaF to enhance the increase in glycogen phosphorylase activity caused by the addition of Ca^{2+} to hepatocytes incubated in the absence of Ca^{2+} , whereas the effects of NaF on O_2 utilization and ATP concentrations were not affected by $AlCl_3$. Moreover, as discussed by Blackmore *et al.* (1985), there is no evidence that the inhibition by F^- of protein phosphatases is potentiated by $AlCl_3$. (4) It has previously been shown (Barritt *et al.*, 1981) that antimycin A and oligomycin, which would be expected to decrease intracellular ATP concentrations (cf. the action of NaF), do not affect plasma-membrane Ca^{2+} inflow, as judged by measurement of the initial rate of $^{45}Ca^{2+}$ exchange.

AlF_4^- has been shown to mimic the action of GTP in

activating isolated GTP-binding regulatory proteins (Sternweiss & Gilman, 1982). Evidence that effects of AlF_4^- on isolated GTP-binding regulatory proteins can be reproduced in intact cells by addition of NaF has been presented (Blackmore *et al.*, 1985; Blackmore & Exton, 1986). Other studies have indicated that in cells the concentration of endogenous Al^{3+} is sufficient to allow the formation of some AlF_4^- , which can activate GTP-binding regulatory proteins (Sternweiss & Gilman, 1982). In the present experiments, the effect of NaF, but not that of vasopressin, on glycogen phosphorylase activity was substantially increased by addition of exogenous AlCl_3 , and inhibited by addition of deferoxamine. On the basis of these observations, it is concluded that the present results provide evidence which indicates that a GTP-binding regulatory protein is involved in the mechanism by which vasopressin stimulates plasma-membrane Ca^{2+} inflow in the liver cell. However, the possibility that some component of the effects of NaF on parameters used to assess Ca^{2+} inflow is due to actions of NaF which are independent of the formation of AlF_4^- cannot be completely excluded.

The putative GTP-binding regulatory protein may stimulate phosphoinositide-specific phospholipase C and hence cause subsequent increases in inositol polyphosphates, $[\text{Ca}^{2+}]_i$ and protein kinase C (Blackmore *et al.*, 1985). Alternatively, the interaction of the GTP-binding regulatory protein with the Ca^{2+} -inflow transporters may occur by a process independent of the activation of phospholipase C (cf. Molski *et al.*, 1984; Pfaffinger *et al.*, 1985). As discussed above, the present results indicate that $[\text{Ca}^{2+}]_i$ and protein kinase C are not involved. The possibilities that the link involves InsP_3 or another inositol polyphosphate or a phospholipase C-independent interaction with Ca^{2+} -inflow transporters require investigation.

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