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# Effect of Islet-Activating Pertussis Toxin on the Binding Characteristics of $Ca^{2+}$-Mobilizing Hormones and on Agonist Activation of Phosphorylase in Hepatocytes

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## SUMMARY

Islet-activating protein (IAP, a *Bordetella pertussis* toxin) was employed to test the hypothesis that the inhibitory GTP-binding regulatory protein of adenylate cyclase ($N_i$) mediates GTP effects on the binding of $Ca^{2+}$-mobilizing hormones to liver plasma membranes and is involved in calcium mobilization stimulated by these agonists. IAP added to normal liver plasma membranes catalyzed the incorporation of radioactivity from [$^{32}$P]NAD into a 41,000-Da peptide (presumably the $\alpha$-subunit of $N_i$). However, no such incorporation was observed in liver membranes prepared from rats 24 hr after intraperitoneal injection of IAP. Angiotensin II attenuated glucagon-stimulated increases in cAMP in hepatocytes prepared from control but not IAP-treated rats. In contrast, following IAP treatment, no changes were observed in the ability of glucagon, vasopressin, angiotensin II, or epinephrine to activate phosphorylase; nor did this treatment alter [$^3$H]vasopressin binding or epinephrine displacement of [$^3$H]prazosin binding. However, IAP treatment decreased [$^3$H]angiotensin II binding affinity when studies were performed in the absence but not the presence of 5′-guanylylimidodiphosphate (GppNHp). This shift was small and represented only 5–8% of the shift in apparent $K_d$ elicited by GppNHp in untreated membranes. *In vitro* studies with IAP confirmed the results of the radioligand binding studies using *in vivo* IAP treatment. The effects of NaCl on [$^3$H]angiotensin II binding were also tested but were not typical of other receptors which couple to $N_i$. The data suggest that, although a small population of hepatic angiotensin II receptors couple to $N_i$ and attenuate glucagon-stimulated increases in cAMP, vasopressin, $\alpha_1$-adrenergic, and the majority of angiotensin II receptors do not interact significantly with $N_i$. Thus, although there is evidence that agonist-induced $Ca^{2+}$ mobilization requires a GTP-binding regulatory protein, this protein does not appear to be $N_i$ in rat liver.

There is now much evidence supporting the theory that hormonal stimulation of intracellular $Ca^{2+}$ mobilization is a consequence of receptor-mediated hydrolysis of $PIP_2$ to $IP_3$ and diacylglycerol (for reviews see Refs. 1–3). Very recent studies on $Ca^{2+}$-mobilizing receptor systems have focused on receptor-mediated events proximal to this $PIP_2$ hydrolysis (4–15). These studies (4–17) indicate that one or more GTP-binding proteins may play a role in agonist-induced hydrolysis of $PIP_2$, in analogy to the role $N_s$ plays in $\beta$-adrenergic- and glucagon-stimulated adenylate cyclase systems (for review see Ref. 18). In some of the abovementioned studies IAP, which catalyzes ADP-ribosylation of $N_i$ and is capable of uncoupling receptors from $N_i$ (see Ref. 19 for review), was found to inhibit receptor-mediated events including $PIP_2$ hydrolysis and $IP_3$ formation (5, 10–12, 15), $Ca^{2+}$ mobilization (6, 8, 10, 11), and physiological events which are dependent on $Ca^{2+}$ mobilization (4, 6, 7, 9–11, 14, 15). This suggests that the GTP-binding regulatory protein(s) which mediates receptor-stimulated $PIP_2$ hydrolysis in the various systems studied may be an IAP substrate such as $N_i$. In contrast, two other reports have appeared which indicate that $PIP_2$ hydrolysis stimulated by muscarinic agonists in cultured heart cells (20) and by thrombin in 3T3 fibroblasts (21) is not inhibited by pertussis toxin. Also, one report has appeared which indicates that $\alpha_1$-adrenergic receptors in rat ventricular myocytes are not linked to a pertussis toxin substrate (22); however, in cultures of these cells the opposite may be true (17).

There are several pieces of evidence which suggest that a GTP-binding protein may mediate EPI, ANG, and AVP stim-

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**ABBREVIATIONS:** $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $IP_3$, *myo*-inositol trisphosphate, EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPI, epinephrine; ANG, angiotensin II (human sequence); AVP, [8-arginine] vasopressin; $N_s$ and $N_i$, respectively, the stimulatory and inhibitory guanine nucleotide-binding regulatory proteins of adenylate cyclase systems; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IAP, islet-activating protein (pertussis toxin); GppNHp, 5′-guanylylimidodiphosphate; DTT; dithiothreitol.

ulation of $IP_3$ release in hepatocytes, namely: 1) the binding of these agonists to their respective $Ca^{2+}$-mobilizing receptors is inhibited by GTP analogs but not other nucleotides (see Ref. 23 for references); 2) fluoride ions stimulate $IP_3$ release, $Ca^{2+}$ mobilization, diacylglycerol accumulation, and phosphorylase activation in liver cells, and aluminum ions enhance this stimulation (16); and 3) GTP analogs regulate $Ca^{2+}$-stimulated and hormone-stimulated $PIP_2$ breakdown and $IP_3$ release from liver plasma membranes (24, 25). Although the identity of the GTP-binding protein which regulates these effects in liver cells is not known, Itoh *et al.* (26) have proposed that $N_i$ or its subunit may mediate hepatic $\alpha_1$-adrenergic events. However, the nature of the putative GTP-binding protein which couples to hepatic angiotensin II and vasopressin receptors is not known.

In the present communication, we have utilized IAP to explore whether $N_i$ is the GTP-binding protein which exerts effects on agonist binding and possibly mediates $Ca^{2+}$ mobilization in hepatocytes exposed to $\alpha_1$-agonists, ANG, and AVP. The data indicate that, whereas a very small population of ANG receptors may be coupled to $N_i$ and capable of inhibiting cAMP formation in liver cells, the $\alpha_1$-adrenergic, AVP, and the majority of ANG receptors do not appear to be coupled to $N_i$ and do not activate phosphorylase by interacting with $N_i$.

## Experimental Procedures

**Materials.** Radiolabeled compounds were obtained from New England Nuclear (Boston, MA). IAP was purchased from List Biological Laboratories (Campbell, CA). Leupeptin and antipain were from Transformation Research (Framingham, MA). Ultra-pure SDS-PAGE reagents were purchased from Idea Scientific Co. (Corvallis, OR). X-ray film (XAR-5) was from Eastman Kodak (Rochester, NY) and Cronex film cassettes with Quanta III intensifying screens (Dupont) were from Picker International (Highland Heights, OH). Sources of other materials have been described previously (16, 23).

**Methods for hepatocyte and liver plasma membrane preparation and radioligand binding studies.** Liver parenchymal cells from male rats (200–210 g) were prepared, and measurements of phosphorylase and cAMP were carried out as described elsewhere (16, 23, 27). The specific binding of [$^3$H]AVP (40–50 Ci/mmol), [$^3$H]ANG (40–65 Ci/mmol), and [$^3$H]prazosin (80 Ci/mmol) to liver plasma membranes prepared with Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was performed and analyzed as detailed elsewhere (23, 28) except that protease inhibitors (leupeptin and antipain, 20 $\mu$g/ml each) were added to the binding buffers. All cell incubations were performed in triplicate and duplicate samples were assayed. Data shown from cell experiments are averaged from three separate studies, whereas data from radioligand binding studies are from single experiments representative of three or more using different batches of membranes. Propranolol (1 $\mu$M) was added in all experiments with EPI to eliminate possible effects mediated by $\beta$-adrenergic receptors.

**IAP-stimulated [$^{32}$P]ADP incorporation into $N_i$.** Liver plasma membranes were prepared simultaneously from control and IAP-injected rats ("*in vivo* IAP treatment," see below). In some experiments, membranes were treated with IAP *in vitro* as described later. In order to determine the extent to which $N_i$ was modified by the treatments (11), IAP-stimulated incorporation of [$^{32}$P]ADP into the 41-kDa subunit of $N_i$ in liver plasma membranes[4] was measured using the assay conditions recommended by Ribeiro-Neto *et al.* (29). For this purpose IAP was activated for 30 min at 30° with 20 mM DTT. The [$^{32}$P]ADP-ribosylation assay mixture contained final concentrations of the following in 250 $\mu$l: 10 $\mu$M [$^{32}$P]NAD (20–40 $\mu$Ci/tube), 50 mM Tris-HCl (pH 7.4); 10 mM thymidine, 1 mM EDTA, 1 mM ATP, 0.1 mM GTP, 20 $\mu$g/ml of leupeptin, 20 $\mu$g/ml of antipain, 50 $\mu$g/ml of activated IAP (carrying over 0.8 mM DTT), and 120 $\mu$g of liver membrane protein. The assay at 30° was started by the addition of liver membranes and terminated after a 20-min incubation by the addition of 1 ml of ice-cold stopping solution containing 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. In some experiments (not shown) an additional 10 $\mu$M [$^{32}$P]NAD (100 $\mu$Ci/tube, final), 1 mM ATP, 0.1 mM GTP was added to control and IAP-treated membranes or cholate extracts at 7-min intervals (see Ref. 9) in a 10-$\mu$l volume. Membranes were sedimented by centrifugation at 10,000 × *g* for 4 min and the supernatant was removed. The membranes were then solubilized by boiling in 150 $\mu$l of 2% SDS, 25% glycerol, 25 mM DTT, 20 mM Tris, 0.15 M glycine buffer (pH 8.2) and 0.03% bromophenol prior to SDS-PAGE in duplicate on 10% acrylamide slab gels as described by Chrisman *et al.* (30). Autoradiographs were prepared from the dried gels. The radioactive content of $N_i$ was determined by slicing the 41-kDa region of the gels into vials containing 10 ml of Beckman Redisolv EP and counting in a Beckman LS 1800 liquid scintillation spectrometer.

***In vivo* treatment with IAP.** Since the time course of IAP on isolated hepatocytes was too slow to make *in vitro* experiments technically feasible, preliminary studies were conducted to evaluate *in vivo* treatment with IAP (31). For this purpose animals were housed individually in metabolic cages with free access to powdered rat chow and water, 48 hr before and 1 week after IAP or sham injections (or until death). Animals were monitored daily for changes in body weight, urine, and fecal output, in addition to changes in body functions and behavior.

No significant [$^{32}$P]ADP incorporation into the 41-kDa peptide [presumably the $\alpha$-subunit of $N_i$ (29)] was observed in liver membranes from animals 24 hr after intraperitoneal injection with nonactivated IAP [50 $\mu$g/100 g of body weight (31)], suggesting that maximal *in vivo* modification of $N_i$ had taken place. Whereas sham-injected rats (198–201 g starting body weight) gained 6–8 g of body weight per day during the observation period, IAP-injected animals gained no significant weight during the first 24 hr and subsequently lost 14–28 g of body weight per day until death.

Twenty-four hr after a lower IAP dose (25 $\mu$g/100 g of body weight), hepatic $N_i$ was modified to the same extent as the higher dose (see Fig. 1) although weight gain was similar to that of control rats. However, at 48 hr, the injected animals either began to lose weight progressively or gained significantly less weight than did paired controls. No other outward signs of toxicity were evident in the injected animals until 24–48 hr before death (when observed). Thus, in order to avoid any effects which might be secondary to decreased food consumption or diminished weight gain, *in vivo* IAP treatments used in this study were for 24 hr only, following injection of 25 $\mu$g of IAP/100 g of body weight.

***In vitro* treatment of liver membranes.** In order to investigate the effects of IAP *in vitro* on radioligand binding parameters of liver plasma membranes, membranes were washed and resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 20 $\mu$g/ml each of leupeptin and antipain. Membranes were then incubated for 20 min at 30° in the presence of final concentrations of the following: 5 mM NAD, 2.5 mM ATP, and 10 mM thymidine plus 300 $\mu$M DTT ("control") or 50 $\mu$g/ml of activated IAP ("IAP-treated," final DTT concentration 300 $\mu$M). Following the 20-min incubation, membranes were diluted with a buffer containing 50 mM Tris-HCl (pH 7.4), 21 mM $MgCl_2$, 2 mM EGTA, and protease inhibitors (20 $\mu$g/ml each of antipain and leupeptin) for use in binding assays or treated as membranes used in binding assays (except without radioligand) for evaluation of the extent to which $N_i$ was modified by the treatment. In these experiments binding buffers were supplemented with 5 mM NAD and 2.5 mM ATP.

**Data analysis.** $K_d$ and $B_{max}$ values from radioligand binding experiments were calculated as previously described (28) and parameters from replicate studies on control and various treatment groups were tabulated for comparative statistical analysis. The data appeared to be normally distributed (Wilks-Shapiro test) and were therefore analyzed for statistical differences by the Student's *t* test at a significance level of 0.05.

[4] Liver membranes or 1% cholate extracts of membranes yielded similar results in these ADP-ribosylation experiments.

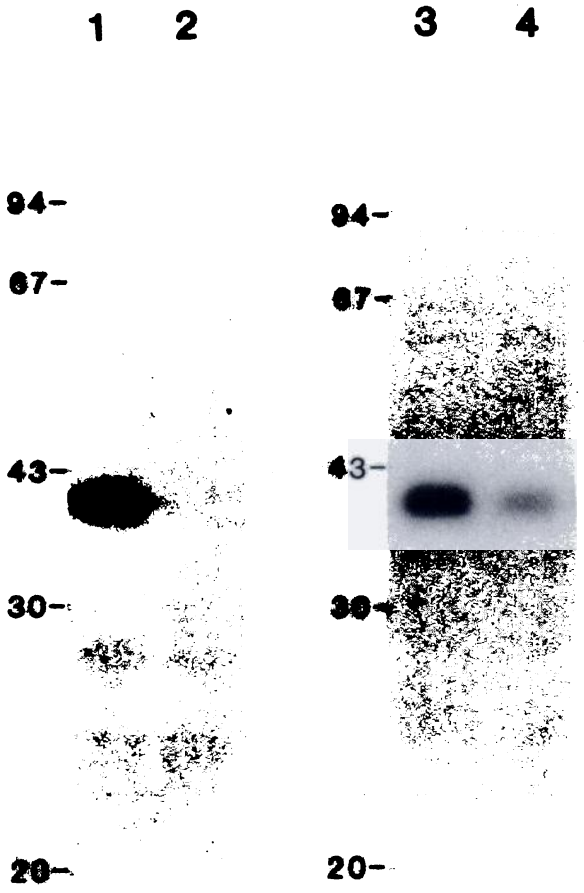


**Fig. 1.** Attentuation of IAP-stimulated [$^{32}$P]ADP-ribosylation of a 41-kDa peptide in liver plasma membranes following IAP treatment *in vivo*. Liver membranes were prepared from control rats (lane *1*) or from rats 24 hr after injection with 25 $\mu$g of IAP/100 g of body wt. (lane *2*). IAP-stimulated [$^{32}$P]ADP-ribosylation of the 41-kDa subunit of $N_i$ in these membranes was measured as described in Experimental Procedures. In another experiment, IAP-stimulated [$^{32}$P]ADP-ribosylation was measured using membranes which had been previously treated without (lane 3) or with (lane *4*) IAP *in vitro* as described in Experimental Procedures. Autoradiographs showing the position of molecular weight standards are shown. These are from typical *in vivo* and *in vitro* IAP treatment experiments and are representative of three separate studies on different membrane batches. Remaining membranes from each batch were used in radioligand binding studies.

## Results

***In vivo* and *in vitro* treatments with IAP.** Liver membranes and hepatocytes were prepared simultaneously from untreated rats or rats treated with IAP (25 $\mu$g/100 g of body weight) 24 hr after injection. Fig. 1 shows that IAP promoted the incorporation of radioactivity from [$^{32}$P]NAD into a 41-kDa peptide in control liver membranes *in vitro*. However, in comparison to control, only 2.2–2.7% of this radioactivity was incorporated into the 41-kDa region of liver membranes from IAP-treated rats.[5] In addition, the ability of ANG to inhibit glucagon-stimulated cAMP formation in hepatocytes from control rats was abolished in hepatocytes prepared from *in vivo* IAP-treated rats (Fig. 2). These data suggest that the extent to which the 41-kDa subunit of $N_i$ was modified by the *in vivo* IAP-treatment is sufficient to abolish hormonal inhibition of cellular cAMP formation.

Conversely, following treatment of liver membranes *in vitro* with IAP, subsequent IAP-mediated [$^{32}$P]ADP-ribosylation of the 41-kDa region of autoradiograms was diminished by only 45–59% in three separate experiments (Fig. 1).

**Effect of IAP and NaCl on radioligand binding parameters.** Hormone receptors coupled to inhibition of adenylate cyclase through $N_i$ typically share some agonist binding characteristics in common. First, IAP treatment elicits a decrease in agonist binding affinity which is not greater than (or additive with) that observed in the presence of maximally effective concentrations of guanine nucleotides (31–35). Second, sodium ions decrease the agonist binding affinity of these receptors and this decrease is additive with that observed with GTP analogs (for review see Ref. 36). We therefore investigated the effects of IAP and NaCl on agonist binding characteristics of AVP, ANG, and $\alpha_1$-adrenergic receptors.

Fig. 3 shows the concentration-dependent specific binding of [$^3$H]AVP and [$^3$H]ANG to membranes from control and IAP-treated rats. As previously reported (23, 37, 38), Scatchard plots (39) of the binding were linear in the present study, yielding correlation coefficients greater than 0.97 (not shown). In contrast, EPI displacement of [$^3$H]prazosin binding yielded biphasic Scatchard plots (not shown), as previously described (28). The majority of the EPI receptor binding sites (63–68%) were in the higher affinity state, called $\alpha_{1H}$ (28), whereas the remaining sites were in the lower affinity form termed $\alpha_{1L}$ (Fig. 3C). GppNHp, a nonhydrolyzable GTP analog, decreased the agonist binding affinity of both AVP and ANG receptors and resulted in the conversion of $\alpha_{1H}$ sites to the $\alpha_{1L}$ form (Fig. 3).

*In vivo* IAP treatment had no effect on [$^3$H]AVP specific binding or EPI displacement of [$^3$H]prazosin (Fig. 3, A and C). However, as noted above, this treatment reduced IAP-stimulated [$^{32}$P]ADP ribosylation of the 41-kDa peptide by 97–98% (Fig. 1) and abolished ANG inhibition of glucagon-stimulated cAMP formation in hepatocytes (Fig. 2). In contrast to the lack of effect on [$^3$H]AVP and EPI binding, *in vivo* IAP treatment resulted in a small but statistically significant decrease in [$^3$H]ANG binding affinity (Fig. 3B) with no effect on $B_{max}$ values. Thus, $K_d$ (nM) and $B_{max}$ (fmol/mg of protein) values for [$^3$H]ANG binding to hepatic plasma membranes were: for control, $K_d = 1.3 \pm 0.1$, $B_{max} = 2190 \pm 60$; and for IAP treated, $K_d = 1.8 \pm 0.2$, $B_{max} = 2140 \pm 130$, averaged from four separate experiments. IAP treatment had no effect, however, on the [$^3$H]ANG binding parameters measured in the presence of 0.5 mM GppNHp, which when averaged from three separate experiments were: for control, $K_d = 9.6 \pm 1.0$, $B_{max} = 1990 \pm 70$; and for IAP treated, $K_d = 9.2 \pm 0.9$, $B_{max}$ 1960 $\pm$ 170. Similar results were observed following the "*in vitro* treatment" of liver membranes with IAP (Table 1, Fig. 4).[6]

The presence of 100 mM NaCl caused statistically significant increases in the apparent $K_d$ values for [$^3$H]AVP and EPI

[5] In order to quantitate incorporation of the very small amount of radioactivity found in the 41-kDa region of gels, additional ATP, GTP, and [$^{32}$P]NAD were added at 7-min intervals as described in Experimental Procedures.

[6] It might be expected *a priori* that the magnitude of the shift in [$^3$H]ANG binding would be greater following the *in vivo* as compared to the *in vitro* pretreatment period. In contrast, they were fairly comparable. The reason for this discrepancy is not clear; however, it may be due in part to the fact that shift in binding is very small to begin with and/or to factors inherent to the different methodologies employed.

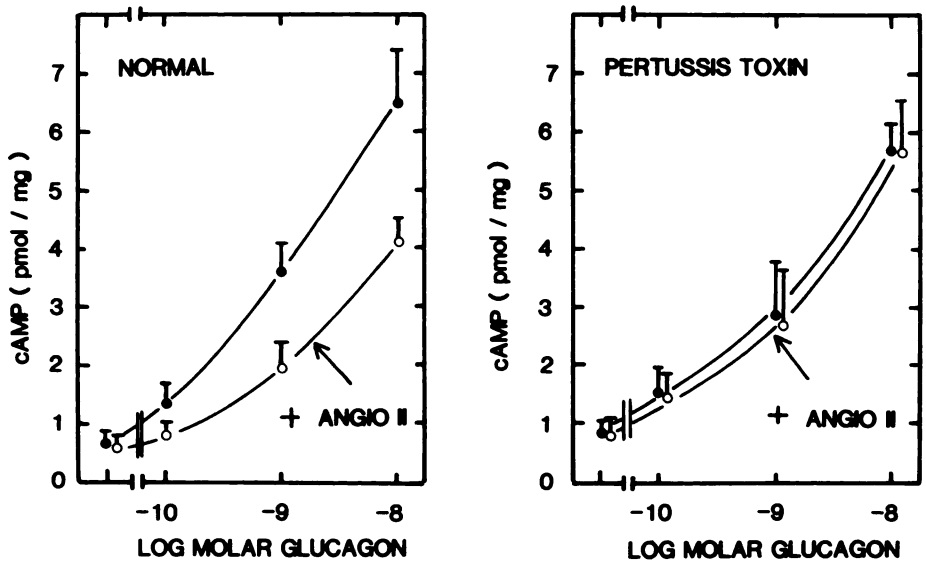


**Fig. 2.** Effect of *in vivo* IAP treatment on ANG inhibition of glucagon elevation of cAMP in rat hepatocytes. Hepatocytes simultaneously prepared from control (*left*) or *in vivo* IAP-treated rats (*right*) were incubated with various concentrations of glucagon in the presence (○) or absence (●) of 10 nM ANG. Aliquots of the cells (0.5 ml) were removed after 5 min of incubation at 37° under an atmosphere of 95% $O_2$/5% $CO_2$ and added to 0.5 ml of 0.6 M $HClO_4$ and stored at −70° until assayed for cAMP content. Results are expressed as pmol of cAMP/mg of wet wt. and each *point* is the mean ± SE from three separate studies.

binding to liver membranes but did not affect $B_{max}$ values. The changes in $K_d$ values for [$^3$H]AVP and EPI induced by $Na^+$ ions were also apparent in the presence of 0.5 mM GppNHp (Table 2). In contrast, NaCl increased [$^3$H]ANG binding affinity in the absence of GppNHp but decreased the affinity in the presence of GppNHp. Thus, NaCl increases the effects of guanine nucleotides on [$^3$H]ANG binding to liver membranes. The presence or absence of NaCl did not change the magnitude of the IAP effect on [$^3$H]ANG binding or alter the lack of effect on the binding of the other $Ca^{2+}$-mobilizing agonists (data not shown).

**IAP and hormonal activation of phosphorylase.** The dose-dependent activation of glycogen phosphorylase by AVP, ANG, EPI, or glucagon was not affected by IAP pretreatment (Fig. 5) in the same batches of cells in which ANG inhibition of glucagon-elevated cAMP formation was abolished (Fig. 1). These data indicate that, if $Ca^{2+}$ mobilization induced by AVP, ANG, and EPI receptors occurs via a GTP-binding protein, this protein is probably not a substrate of pertussis toxin such as $N_i$.

## Discussion

The initial interaction of EPI, ANG, and AVP with liver cells appears to be the binding of these agonists to their respective specific receptors on the plasma membrane surface. There is much evidence that, as a consequence of this receptor occupancy, $Ca^{2+}$ is rapidly mobilized from intracellular stores leading to a rise in free cytosolic [$Ca^{2+}$] and subsequent activation of glycogen phosphorylase and other enzymes (2, 3). Until recently, the series of events which occurs following stimulation of $\alpha_1$-adrenergic, AVP, and ANG receptors was poorly understood. It is now widely accepted that the mobilization of $Ca^{2+}$ from intracellular stores in liver cells (2, 3) as well as other tissues (1) is a result of the receptor-mediated breakdown of $PIP_2$ to $IP_3$ and diacylglycerol, and many investigators have now begun to turn their attention to the events which occur prior to $PIP_2$ hydrolysis (4–17; see Ref. 1 for review). Data from these more recent studies (4–17, 24) indicate that a GTP-binding regulatory protein may be involved in transducing occupancy of a $Ca^{2+}$-mobilizing receptor into activation of a $PIP_2$-specific phosphodiesterase (phospholipase C).

In liver cells there are three lines of evidence which suggest that a GTP-binding regulatory protein is involved in receptor-mediated $Ca^{2+}$ signaling. These are: 1) the finding that guanine nucleotides decrease the agonist binding affinity of AVP-, ANG-, and $\alpha_1$-adrenergic receptors (28, 37, 38; for example, see Fig. 3) in a manner analogous to that observed in $\beta$-adrenergic, opiate, $\alpha_2$-adrenergic, and other receptors which are coupled to adenylate cyclase through $N_s$ and $N_i$ (31–33; for review see Ref. 36); 2) the recent data of Blackmore *et al.* (16) showing that several events which are dependent on $PIP_2$ hydrolysis in liver cells may be stimulated by fluoride ions and that this fluoride effect is potentiated by aluminum ions; and 3) the observation that GTP and GTP analogs synergistically increase hormone- and $Ca^{2+}$-stimulated breakdown of labeled $PIP_2$ and release of inositol phosphates from liver membranes previously labeled with [$^3$H]*myo*-inositol (24, 25).

Although the identity of the GTP-binding regulatory protein(s) which mediates the above effects in liver is not known, data from other tissues indicate that $N_i$ or a closely related protein may be involved in transducing receptor-mediated $PIP_2$ hydrolysis and, ultimately, the events which are secondary to $PIP_2$ hydrolysis (5, 6, 8–11, 14, 15, 17). In these studies (5, 6, 8–11, 14, 15, 17), IAP was employed. IAP inactivates $N_i$ by catalyzing ADP-ribosylation of the $\alpha$-subunit (41 kDa) using NAD as donor (see Ref. 19 for review). Following this action of IAP, receptors coupled to $N_i$ are unable to form high affinity agonist complexes (31–34) and are incapable of signaling (see Ref. 19 for review). Since IAP has now been shown to interrupt events in several systems which are thought to rely on $PIP_2$ hydrolysis, and since the percentage of attenuation of $Ca^{2+}$-signaling can be correlated to the percentage of modification of $N_i$, it is reasonable to hypothesize that $N_i$ may regulate $PIP_2$ hydrolysis in these systems (5, 6, 8–11, 14, 15, 17).

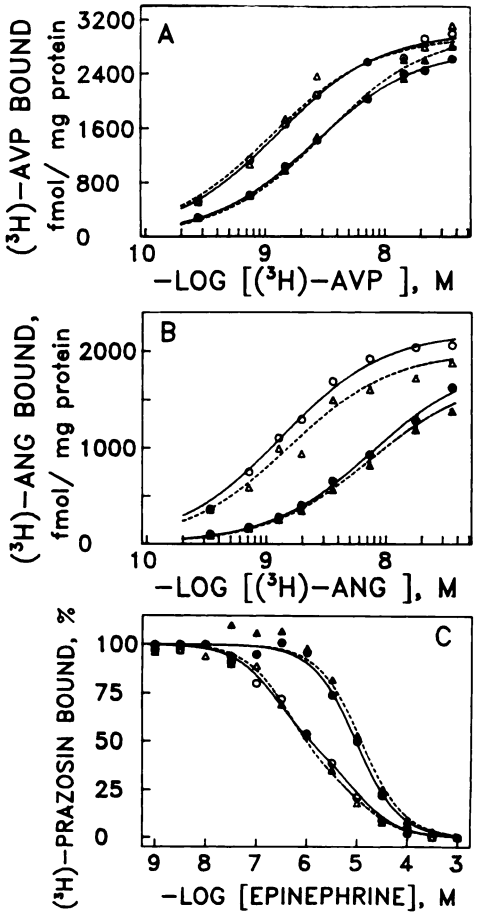


**Fig. 3.** Effect of *in vivo* IAP treatment on the binding of $Ca^{2+}$-mobilizing agonists to liver plasma membranes. Concentration-dependent binding of [$^3$H]AVP (A), [$^3$H]ANG (B) or EPI displacement of 1 nM [$^3$H]prazosin (C) to liver plasma membranes from control (○, ●) or *in vivo* IAP-treated (△, ▲) rats was measured in the presence (*solid symbols*) or absence (*open symbols*) of 0.5 mM GppNHp as described in Experimental Procedures. The data were calculated, curve-fitted, and drawn using MLAB as previously described (28). Each *point* is the mean from triplicate determinations and each *panel* is representative of three to four such studies.

TABLE 1

**Lack of effect of *in vitro* IAP treatment on [$^3$H]AVP binding and EPI displacement of [$^3$H]prazosin**

*In vitro* IAP treatments were performed as described in Experimental Procedures; binding of [$^3$H]AVP (8 concentrations) and EPI (14 concentrations) was performed in the presence of 1 nM [$^3$H]prazosin as in Fig. 3. $B_1$ and $B_2$ are the binding capacities of the high and low affinity $\alpha$-adrenergic binding sites, respectively, and were determined as previously described (28). The results shown are representative of three studies with each radioligand. None of the results for IAP-treated membranes were significantly different from control as determined by the Student's *t* test at a significance level of 0.05.

| Ligand | Binding parameter | Control membranes | IAP-treated membranes |
|---|---|---|---|
| [$^3$H]AVP | $B_{max}$ (fmol/mg of protein) | 3270 ± 130 | 3210 ± 130 |
| | $K_d$ (nM) | 1.5 ± 0.3 | 1.7 ± 0.3 |
| EPI | $B_1$ (%) | 57 ± 5 | 59 ± 6 |
| | $B_2$ (%) | 43 ± 6 | 41 ± 7 |
| | $K_d$ ($\alpha_{1H}$, nM) | 23 ± 4 | 17 ± 6 |
| | $K_d$ ($\alpha_{1L}$, nM) | 600 ± 120 | 740 ± 50 |

Since the role of $N_i$ in this regard may not be universal (20, 21), we initiated a study of IAP effects on the specific binding of $Ca^{2+}$-mobilizing hormones to liver plasma membranes and on the ability of AVP, ANG, and EPI to activate glycogen

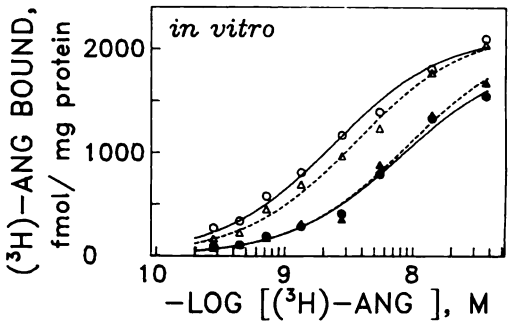


**Fig. 4.** Effect of *in vitro* IAP treatment on the binding of [$^3$H]ANG to liver plasma membranes. Membranes were control (○, ●) or *in vitro* IAP-treated (△, ▲) as described in Experimental Procedures. [$^3$H]ANG binding to these membranes was measured exactly as in the legend to Fig. 3 in the presence (●, ▲) or absence (○, △) of 0.5 mM GppNHp.

TABLE 2

**Differential effect of NaCl on the dissociation constants for agonist binding to $Ca^{2+}$-mobilizing receptors of rat liver plasma membranes**

Dissociation constants were measured as described in Experimental Procedures. Experiments are shown which are representative of three studies with each ligand. Final concentrations of the indicated additions were: sucrose, 170 mM; NaCl, 100 mM; GppNHp, 0.5 mM. None of the different conditions significantly affected $B_{max}$ values, and the addition of sucrose had no effect on binding parameters. EPI displacement of [$^3$H]prazosin binding was measured as in Fig. 3. $Na^+$ reduced the $K_d$ for [$^3$H]prazosin from 88 to 135 pM. A similar small increase was observed in two other studies. $K_d$ values for EPI were obtained as described in detail elsewhere (28). $Na^+$ did not effect the ratio of $\alpha_{1H}$ to $\alpha_{1L}$ receptors measured in the absence of GppNHp.

| Ligand | Assay condition | $K_d$ ± SE | $\frac{K_d + GppNHp}{K_d - GppNHp}$ Ratio |
|---|---|---|---|
| | | nM | |
| [$^3$H]AVP | Sucrose | 1.2 ± 0.2 | 3.4 |
| | Sucrose and GppNHp | 4.1 ± 0.6 | |
| | NaCl | 1.7 ± 0.1* | 3.4 |
| | NaCl and GppNHp | 5.8 ± 0.8* | |
| [$^3$H]ANG | Sucrose | 3.1 ± 0.4 | 1.8 |
| | Sucrose and GppNHp | 5.7 ± 0.2 | |
| | NaCl | 1.3 ± 0.2* | 7.3 |
| | NaCl and GppNHp | 9.6 ± 1.0* | |
| EPI | Sucrose-$\alpha_{1H}$ | 26 ± 7 | 27 |
| | Sucrose and GppNHp | 714 ± 59 | |
| | NaCl – $\alpha_{1H}$ | 57 ± 14* | 26 |
| | NaCl and GppNHp | 1470 ± 150* | |

* Significantly different from respective "sucrose" or "sucrose and GppNHp" control, $p < 0.05$.

phosphorylase in hepatocytes (an $IP_3$-dependent event; for review see Refs. 2, 3). Both *in vitro* and *in vivo* IAP treatments were tested since both have their advantages and disadvantages. The advantage of *in vitro* IAP treatment is that it takes less time and avoids any possible systemic effects and epiphenomena arising from *in vivo* injection. The *in vitro* approach also allows for an internal control, since control and IAP treatments can be performed on the same batch of liver plasma membranes. The disadvantages for our purposes are: 1) the time course of IAP action on intact hepatocytes is too slow to make it an effective pharmacological tool *in vitro*, and 2) the *in vitro* treatment was only partially effective at modifying the $N_i$ population of liver plasma membranes. In contrast, *in vivo* treatment approaches complete modification of the $N_i$ population in liver plasma membranes and allowed us to perform intact cell experiments.

*In vivo* IAP treatment did not inhibit the formation of the higher affinity AVP and $\alpha_{1H}$-adrenergic sites observed in the

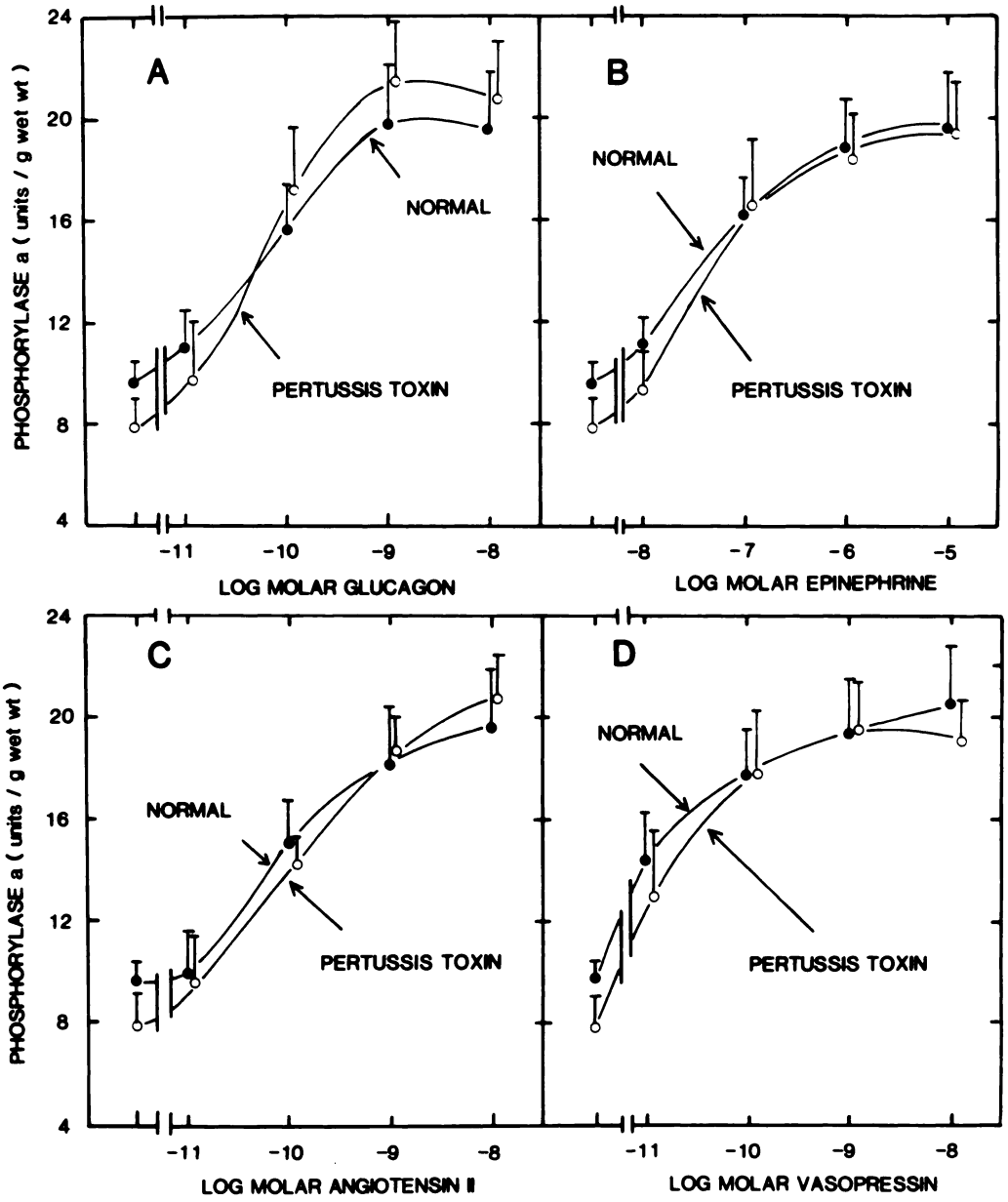


**Fig. 5.** Lack of effect of *in vivo* IAP treatment on hormonal activation of glycogen phosphorylase. Concentration-dependent activation of phosphorylase by glucagon (A), EPI (B), ANG (C), and AVP (D) was measured in hepatocytes prepared from control (●) or *in vivo* IAP-treated (○) rats as previously described (16, 23, 27).

absence of GppNHp but produced a significant decrease in the apparent affinity for [$^3$H]ANG in the absence, but not the presence, of GppNHp (Fig. 3). *In vitro* IAP treatment confirmed the *in vivo* treatment results (Fig. 4, Table 1). *In vivo* IAP treatment appeared to have modified more than 97% of the hepatic $N_i$ (Fig. 1), resulting in complete attenuation of the ability of ANG to lower cAMP elevated by glucagon (Fig. 2). Under these conditions, the concentration-dependent ability of ANG, EPI, and AVP to activate phosphorylase was intact, however (Fig. 5). In addition, no effect of IAP has been observed on vasopressin-stimulated, GTP-dependent $PIP_2$ breakdown in liver membranes following *in vivo* IAP pretreatment (25). This suggests that the inhibition of cAMP formation by ANG occurs through an interaction with $N_i$, but the action of ANG, AVP, and EPI to increase $IP_3$ and mobilize $Ca^{2+}$ (23) may not involve this protein.

Some caution is warranted, however, since for this interpretation we are relying on the enzymatic activity of IAP, which may be modulated by various factors, not the least of which is the conformation and/or configuration of the target protein. For instance, Ribeiro-Neto *et al.* (29) have shown that $Mg^{2+}$ and high phosphate concentrations, which are typically used with cholera toxin, inhibit ADP-ribosylation of $N_i$ by IAP, whereas GTP and ATP stimulate ADP-ribosylation by IAP in the absence of $Mg^{2+}$. They suggest that IAP may "prefer the conformation of $N_i$ which exists when receptors uncouple" (29). In view of this, in the present study we employed the conditions recommended by Ribeiro-Neto *et al.* (29) for our [$^{32}$P]ADP-ribosylation assays.

Another difficulty, however, which is not addressed in the present study comes from the findings of Neer *et al.* (40) who have shown that the activity of IAP toward GTP-binding

proteins in bovine brain is poor in the absence of a 35-kDa β-subunit of the proteins. It might be expected, therefore, that the activity of IAP toward our liver membrane preparation might also be dependent on the concentration of β-subunits. This factor was not controlled in the present study but may be important. For instance, if only free $\alpha_i$ (the α-subunit of $N_i$) were required for coupling $Ca^{2+}$-mobilizing receptors to $PIP_2$ hydrolysis in liver and the concentration of 35-kDa β-subunits in liver were low, IAP might be ineffectve due to the absence of sufficient β-subunits. Alternatively, even if we assume that sufficient β-subunits were present and that IAP was as effective as our study seems to indicate (Figs. 1 and 2), it is still possible that the GTP-binding protein which controls polyphosphoinositide hydrolysis in liver is $N_i$. For example, IAP prevents the dissociation of the $\alpha_i$ and $\beta/\gamma$ subunits of $N_i$ (19) which appears to be required for inhibition of adenylate cyclase, but theoretically might not be required for polyphosphoinositide hydrolysis to occur. Such a model, however, would represent a considerable departure from our current understanding of the *modus operandi* of these regulatory proteins.

Another problem which arises from the present study concerns the fact that IAP inhibited ANG inhibition of cAMP formation (Fig. 2) but did not affect ANG stimulation of phosphorylase activation (Fig. 5). It is possible to hypothesize that ANG is capable of interacting with two distinct subtypes of ANG receptors on liver membranes which separately control adenylate cyclase inhibition and $PIP_2$ hydrolysis. Interestingly, [$^{125}$I]ANG, unlike[$^3$H]ANG, appears to differentially recognize two sites in liver membranes with different $K_d$ values for [$^{125}$I]ANG (41, 42) and differential susceptibility to DTT (41). However, the situation is complicated by the fact that the binding is altered by guanine nucleotides (42). Furthermore, DTT effects on binding (37) could be secondary to modifications, not only of the ANG receptors, but also of the GTP-binding protein(s) required for formation of high affinity agonist complexes.

IAP decreased [$^3$H]ANG affinity significantly following maximal modification of $N_i$ in the present study. However the decrease was small [$K_d$ ratio (IAP-treated/control) = 1.4, Fig. 3B], and only 6% of the magnitude of the shift observed in the presence of a maximally effective concentration of GppNHp [$K_d$ ratio (+GppNHp/−GppNHp) = 7.4, Fig. 3B], which presumably uncouples the rest of the GTP-binding proteins from these receptors. Based on these data, if two ANG receptor subtypes do indeed exist and control distinct physiological events, it would seem that the proportion of the receptors which are coupled to inhibition of adenylate cyclase is quite small. In support of this, the net effects of NaCl on ANG receptor binding parameters were not typical of other hormone receptors which inhibit adenylate cyclase (Table 2). NaCl has inhibitory effects on the binding of agonists that inhibit this enzyme, which are thought to be exerted at the level of the receptor (see Ref. 36 for review). In contrast, NaCl decreased the apparent $K_d$ for [$^3$H]ANG and increased the magnitude of the guanine nucleotide effect on [$^3$H]ANG binding.

An alternative to the hypothesis that two distinct populations of ANG receptors exist in rat liver which control either inhibition of adenylate cyclase or $PIP_2$ hydrolysis is the possibility that a single population of ANG receptors exists which can couple either to $N_i$ or the GTP binding protein(s) involved in $Ca^{2+}$ signaling.[7] In liver, the concentration of $N_i$ may be low relative to the binding protein involved in $Ca^{2+}$ mobilization or there may be differences in the receptor coupling efficiency of these proteins such that the major effect of ANG is to elevate $Ca^{2+}$ rather than lower cAMP. Observations with intact hepatocytes do in fact show that ANG induces no detectable decrease in cAMP in the absence of agonists which stimulate adenylate cyclase (Fig. 2 and Ref. 44).

In conclusion, we have employed IAP to test the hypothesis that $N_i$ mediates $Ca^{2+}$ mobilization by AVP, ANG, and EPI in rat liver. The observations that IAP can prevent ANG inhibition of cAMP formation and that, under these conditions, receptor binding and phosphorylase activation by EPI and vasopressin are unaffected appear to suggest that $N_i$ is not involved in hormonally mediated $PIP_2$ hydrolysis in liver.

[7] In support of this possibility, it has been observed that phorbol esters attenuate fluoride stimulation of phosphorylase and $IP_3$ formation but *not* fluoride inhibition of cyclic AMP formation in liver cells (P. F. Blackmore and J. H. Exton, submitted for publication).

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