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Formation of the high-affinity agonist state of the α_1-adrenergic receptor at cold temperatures does not require a G-protein

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Two methods were employed to uncouple hepatic α_1-adrenergic receptors from their associated G-protein (termed G_p) in order to determine whether locking of the α_1-receptor in a high-affinity agonist state at cold temperatures (2°C) represents formation of a ternary complex. Uncoupling is defined as the inability to observe the GppNHp-sensitive, high-affinity agonist state of the receptor in [^{3}H]prazosin competition binding studies performed at 25°C. The first method for achieving uncoupling involved brief alkalinization and resulted in greater than 95% loss of several G-proteins. The second method involved proteolytic cleavage of either part or all of the α_1-receptor coupling domain from the binding domain. Following either treatment, receptors were converted to the high-affinity agonist state at 2°C. Thus, while formation of the high-affinity state of the receptor at higher temperatures may require G_p, formation of this state at 2°C does not require G_p or even the entire α_1-adrenergic receptor.

α_1-Adrenergic receptor; G-protein; Receptor affinity state; Competition binding; Conformational change; (Liver)

1. INTRODUCTION

In liver cells, α_1-adrenergic receptors stimulate polyphosphoinositide phospholipase C resulting in the formation of the second messengers inositol trisphosphate and diacylglycerol. There is now evidence that an IAP-insensitive GTP-binding regulatory protein (G-protein) termed G_p is involved in regulating the activation of phospholipase C (review [1]). Further evidence for this idea comes from studies which show that guanine nucleotides alter agonist affinity at α_1-adrenergic receptors in competition radioligand-binding experiments [2–8] by analogy with β-adrenergic receptors [9]. When these binding studies are conducted at 25–37°C, α_1-adrenergic receptor populations display two affinities for agonists and a single affinity for antagonist. A number of guanine nucleotides are capable of converting the higher affinity receptors to the low-affinity state [2–8]. It has been proposed that in analogy to β-adrenergic receptors, the high-affinity state represents the formation of a ternary complex of agonist, receptor and G_p [9]. According to the ternary complex theory proposed by Delean et al. [10], the formation of this complex is agonist-induced and is presumably required for the stabilization of the high-affinity configuration.

Our laboratory has previously reported that incubation of liver plasma membranes at 2°C converts α_1-adrenergic receptors into a homogenous population of sites which has a high affinity for

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Abbreviations: IAP, islet-activating protein, a *Bordetella pertussis* toxin; PIP_2, phosphatidylinositol 4,5-bisphosphate; G_p, the hepatic GTP-binding regulatory protein which couples Ca^{2+}-mobilizing hormone receptors to PIP_2-phospholipase C; G_s and G_i, respectively, the stimulatory and inhibitory GTP-binding regulatory proteins of adenylate cyclase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; GppNHp, 5′-guanyl imidodiphosphate

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agonists which is insensitive to guanine nucleotides [6]. The K_d of this state for agonists is similar to the high-affinity state observed at higher temperatures [6,9] which presumably reflects the formation of the ternary complex. However, it was unclear from our study [6] or the subsequent study by Schwarz and co-workers [9] whether the high-affinity state observed at 4°C represented the ternary complex. Here, two methods were employed to uncouple hepatic α_1-adrenergic receptors from their associated G-protein (G_p) in order to determine whether locking of the α_1-receptor in an agonist high-affinity state (K_d = 32–56 nM for epinephrine) at cold temperatures (2–4°C) represents formation of a ternary complex. Uncoupling is defined as the inability to observe the GppNHp-sensitive, high-affinity agonist state of the receptor in competition binding studies with [^{3}H]prazosin performed at 25°C. Under uncoupling conditions, only the low-affinity agonist form (K_d = 565 ± 52 nM for epinephrine) of the α_1-receptor is observed and antagonist binding is not altered.

One method for uncoupling involves global removal of G-proteins from the plasma membrane by alkalinization and the other involves proteolysis of the α_1-adrenergic receptor coupling domain from the binding domain. Following either treatment only the low-affinity agonist form of the α_1-adrenergic receptor could be observed at 25°C, although the receptors could be completely converted to the high-affinity agonist form by cold temperature incubation. Thus, while formation of the high-affinity form of the receptor at higher temperatures may be indicative of the 'ternary complex', formation of this state at low temperatures does not require a G-protein or even the complete α_1-adrenergic receptor.

2. EXPERIMENTAL

2.1. *Materials*

Radiolabelled compounds were from New England Nuclear/Dupont (Boston, MA). Leupeptin and antipain were from Transformation Research (Framingham, MA). Sources of other materials have been described previously [6,8,11].

2.2. *Membrane preparation and [^{3}H]prazosin-binding studies*

Liver plasma membranes were prepared from male Sprague-Dawley rats (200–250 g) and [^{3}H]prazosin binding performed as described [6,8,11]. Computer analysis of binding data was performed as detailed in [6] using the mathematical modelling and curve-fitting capabilities of the RS-1 program (BBN Software, Cambridge, MA). In some experiments membranes prepared with 1 mM EGTA and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml antipain [8]) were alkalinized by resuspension in ice-cold medium containing 25 mM Na_2HPO_4 (pH 11.9) and 1 mM EDTA for 45 min on ice with intermittent vortex-mixing [12,13]. The alkaline mixture was centrifuged at 30000 × g for 10 min and the membrane pellet was resuspended in binding buffer (pH 7.4) with 1 mM EGTA and protease inhibitors for use in various assays. These membranes are termed 'alkalinized' membranes in the text.

In other experiments, 4 mM $CaCl_2$ was added and EGTA and protease inhibitors were omitted from the buffer used to wash and resuspend membranes following their isolation on the Percoll gradient. Prior to use, these membranes were incubated for 30 min at 25°C in order to allow proteolysis of the α_1-receptor to occur [8]. Subsequently, the membranes were diluted 30-fold in medium containing EGTA and protease inhibitors, washed by centrifugation (30000 × g × 10 min) and resuspended for use in various assays. These membranes are termed 'Ca^{2+}-treated' in the text.

2.3. *IAP-stimulated [^{32}P]ADP ribosylation and assay of PIP$_2$-specific phospholipase C activity*

IAP-stimulated [^{32}P]ADP incorporation into the α-subunit of liver plasma membrane G_i was performed with repetitive addition of [^{32}P]NAD, ATP and GTP as in [11]. PIP_2-specific phospholipase C activity was measured in liver plasma membranes by measuring release of trichloroacetic acid-soluble radioactivity from exogenous [^{3}H]PIP_2, according to Taylor and Exton [14].

2.4. *[^{32}P]GTP photoaffinity labelling*

For [^{32}P]GTP photoaffinity labelling experiments, 20 μl pre-warmed (30°C) liver membranes (3–6 mg protein/ml) were added to 20 μl pre-warmed media in quartz tubes containing final concentrations of the following: 50 mM Na-Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP and 0.2 μM [α-^{32}P]GTP (3000 Ci/mmol). Non-specific binding was measured in the presence of 100 μM GTPγS, GppNHp or GTP and gave similar results. Following a 1 min reaction the tubes were removed to ice and placed for 3 min (4°C) in a Rayonet RPR-100 Photo Chemical Reactor (Southern New England UltraViolet, Hamden, CT) containing 16 lamps in a circular configuration generating 8 W per lamp of 2537 Å ultraviolet light. The tubes were placed in the reactor (1.5–2 inches from the lamp plane) on a rotating (5 rpm) carrier in order to ensure equal radiation of the samples. Subsequently, the samples were diluted to 1 ml in water and transferred to Eppendorf tubes for precipitation with trichloroacetic acid (10% final concentration). The precipitated proteins were solubilized and boiled in SDS-PAGE sample buffer prior to electrophoresis and autoradiography of the dried gels as in [8,11].

3. RESULTS AND DISCUSSION

3.1. *Effect of membrane treatments on GTP-binding proteins and PIP$_2$-phospholipase C*

Citri and Schramm [12] reported that brief

alkalinization of turkey erythrocyte membranes resulted in the inactivation of adenylate cyclase and G_s activity, but did not damage the β-adrenergic receptor. Similarly, alkalinization of platelet membranes resulted in loss of IAP-stimulated radiolabelling of the α-subunit of G_i but did not significantly alter [^{3}H]antagonist binding to inhibitory α_2-adrenergic receptors [13]. In preliminary experiments, we characterized the effect of brief alkalinization on liver plasma membrane G-proteins. Fig.1 (lanes 1,2) shows that brief alkalinization resulted in a greater than 95% loss in the ability of IAP to incorporate [^{32}P]ADP into the 41 kDa α-subunit of G_i.

Following UV light exposure, [α-^{32}P]GTP became covalently attached to a number of liver plasma membrane peptides in the presence of 5 mM ATP (fig.1, lane 3). In the absence of high concentrations of ATP a greater number of sites were radiolabelled and no labelling was observed without UV light exposure or in the presence of 1% cholate (not shown). Photoaffinity labelling by [α-^{32}P]GTP to 40–49 kDa liver membrane peptides (which may include the α-subunits of G-proteins such as G_s, G_i and G_p) could be abolished by excess GTP, GDP (not shown) or GTPγS (fig.1, lane 4). These findings, plus the fact that the labelling was observed in the presence of a high concentration of ATP, indicate that the photoaffinity radiolabelling resulted from the interaction of [α-^{32}P]GTP with guanine nucleotide-binding sites. In agreement with the IAP-stimulated [^{32}P]ADP-ribosylation results, extensive loss of [α-^{32}P]GTP photoaffinity labelling was observed in alkalinized membranes (fig.1, lanes 5,6).

In order to measure specifically the IAP-insensitive G-protein which couples to Ca^{2+}-mobilizing hormone receptors, a functional assay was employed, namely GTPγS stimulation of PIP_2-phospholipase C activity (table 1). Ca^{2+}-stimulated PIP_2-phospholipase activity was also measured as an index of the membrane content of this enzyme. GTPγS-stimulated phospholipase C activity was abolished by alkalinization. Since the Ca^{2+}-stimulated activity was also abolished, loss of the GTPγS activity cannot be attributed necessarily to inactivation or loss of G_p. Albeit the data from fig.1 and previous studies [12,13], and the fact that α_1-receptors are 'uncoupled' following alkalinization (fig.2, see below),

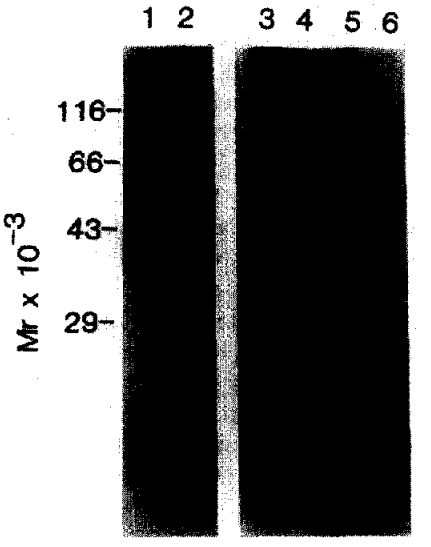

Fig.1. Effect of alkalinization on liver plasma membrane G-proteins. Autoradiographs are shown with the positions of the M_r standards indicated. IAP-stimulated [^{32}P]ADP-ribosylation was measured using control (lane 1) and alkalinized membranes (lane 2). [α-^{32}P]GTP photoaffinity labelling of control (lanes 3,4) and alkalinized (lanes 5,6) membrane peptides was accomplished as described in section 2. Membranes were incubated in the presence (lanes 4,6) or absence (lanes 3,5) of 100 μM GTPγS for determination of non-specific radiolabelling.

would tend to suggest that both G_p (indeed several G-proteins) and PIP_2-specific phospholipase C are either inactivated, lost or both following alkalinization.

We and others have previously described the mechanism of the uncoupling effects of Ca^{2+} on the liver plasma membrane α_1-adrenergic receptor [6,8,15]. This treatment had no significant effect on GTPγS and Ca^{2+}-stimulated PIP_2-specific phospholipase C (table 1) suggesting that, unlike the α_1-adrenergic receptor, these components are not a significant substrate for the endogenous Ca^{2+}-sensitive protease that is co-isolated with the liver plasma membranes.

3.2. *Effect of membrane treatments on α_1-adrenergic receptor specific binding parameters*

Fig.2 (top panel) shows the typical pattern of epinephrine competition for [^{3}H]prazosin binding to α_1-adrenergic receptors at 25 and 4°C described previously [1,6–9,11,15]. Computer analysis of the

Table 1

Effect of various membrane treatments on PIP_2-phospholipase C activity[a]

Membrane treatment	Assay conditions (nmol [^{3}H]PIP_2 hydrolysed/mg protein per min)		
	0.2 μM Ca^{2+}	0.2 μM Ca^{2+} plus 10 μM GTPγS	100 μM Ca^{2+}
Control	0.82 ± 0.25	4.73 ± 1.80	4.75 ± 0.32
Alkalinized	N.A.	N.A.	N.A.
Ca^{2+}-treated	0.96 ± 0.35	3.27 ± 1.33	6.47 ± 0.94

PIP_2-phospholipase C activity was measured in liver plasma membranes (25 μg protein/tube) according to Taylor and Exton [14] using exogenous [^{3}H]phosphatidylinositol 4,5-bisphosphate (0.1 mM, 697–812 cpm/nmol). Assays were for 15 min at 37°C. The data are means ± SE from 3 separate experiments with different membrane preparations. N.A., no activity detected

data [2,6,9,15] indicates that the receptors are converted by GppNHp from a mixed population with higher affinity for epinephrine (61 ± 2.4% of the sites had a K_d = 30 ± 9) to a homogeneous population of sites with a single low affinity for epinephrine (K_d = 637 ± 70 nM). It is presumed that the high-affinity state represents formation of a ternary complex of agonist receptor and G_p [9] by analogy with β-adrenergic receptors [10].

Fig.2 (middle panel) shows that alkalinization produced changes consistent with the uncoupling of α_1-receptors from G_p, since no high-affinity receptors were observed at 25°C in the absence of GppNHp. Instead, epinephrine interaction with α_1-adrenergic receptors from alkalinized membranes could be described as binding to a homogeneous population of sites with a single low affinity for epinephrine (591 ± 88 nM). GppNHp did not cause a further rightward shift in the binding. Similar effects of alkalinization on membrane β- and α_2-adrenergic receptors have been reported previously [12,13]. Fig.2 (bottom panel) shows that incubation of liver plasma membranes with Ca^{2+} also resulted in an apparent uncoupling of α_1-adrenergic receptors from G_p [6,8,15]. This appears to be due to the ability of Ca^{2+} to stimulate a leupeptin/antipain-sensitive protease co-isolated with liver plasma membranes [8,15]. Stimulation of this protease activity by Ca^{2+}

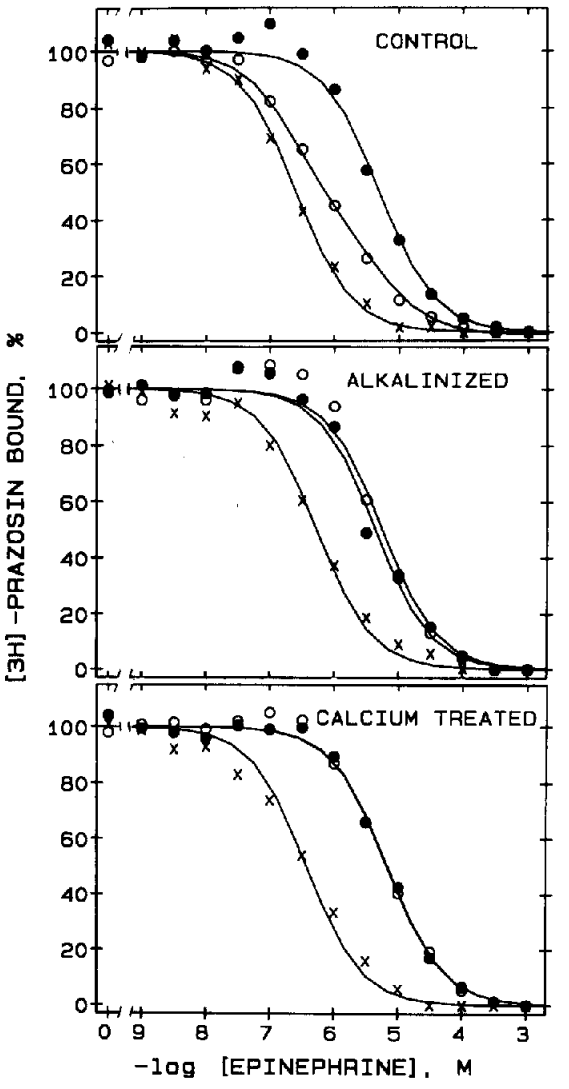

Fig.2. Effect of different membrane treatments on epinephrine competition for [^{3}H]prazosin binding to α_1-adrenergic receptors. Equilibrium [^{3}H]prazosin binding to control (top), alkalinized (middle) and Ca^{2+}-treated (bottom) membranes was measured in the presence of various concentrations of epinephrine as described [6,8,11]. Tubes were incubated at 25°C for 30 min (○,●) or 2°C for 3 h (×) in the presence (●) or absence (○,×) of GppNHp. The results shown are from a single experiment which is representative of two such studies.

results in cleavage of the 78 kDa form of the α_1-adrenergic receptor to lower molecular mass forms ranging from 15 to 59 kDa [8,15]. This treatment has no significant effect on the apparent K_d or B_{max} for [^{3}H]prazosin binding [6,8]. Further-

more, the affinity of epinephrine in the presence of GppNHp is not significantly altered by this treatment. However, as seen in fig.2 (bottom panel), this treatment does result in uncoupling of the α_1-adrenergic receptors since no high-affinity agonist binding is observed at 25°C in the absence of GppNHp [6,8,15]. The apparent K_d for epinephrine in Ca^{2+}-treated membranes at 25°C in the absence of GppNHp was 709 ± 43 nM (fig.2, bottom panel).

3.3. *Effect of cold temperature incubation on α_1-adrenergic agonist binding*

Fig.2 shows that incubation at 2–4°C converts α_1-adrenergic receptors to a homogeneous population of sites with a single high affinity for epinephrine (32 ± 3 nM). Since guanine nucleotides do not affect this high-affinity configuration, it is stated that the α_1-adrenergic receptors are 'locked' [6,9] into a high-affinity state at cold temperatures. Fig.2 shows further that incubation at 2°C resulted in conversion of the receptors to a homogeneous population of high-affinity sites even when they had been uncoupled by the two membrane treatments described above. The apparent K_d values measured at cold temperature were 32 ± 3, 56 ± 5, and 39 ± 5, respectively, for control, alkalinized and Ca^{2+}-treated membranes. Since α_1-adrenergic receptors are not capable of coupling to G_p in alkalinized or Ca^{2+}-treated membranes (as shown by the binding data at 25°C; fig.2), our data indicate that the high-affinity α_1-agonist binding observed at cold temperatures is not representative of a ternary state of agonist, receptor and G_p. This conclusion is further supported by studies on α_1-adrenergic receptors of BC_3H_1 [17] and DDT_1MF-2 [18] cells. In these cells high-affinity α_1-adrenergic agonist binding is observed at 2–4°C, despite the fact that the α_1-adrenergic receptors appear to be uncoupled (i.e. no guanine nucleotide-sensitive high-affinity agonist binding is observed in equilibrium binding experiments). Since Ca^{2+} treatment results in proteolysis of α_1-adrenergic receptors, our data also indicate that the formation of the high-affinity agonist state at cold temperatures does not require the entire α_1-adrenergic receptor and therefore may be a function of the binding domain itself.

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