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Absence of a direct coupling of a G protein to dihydropyridine binding sites in rat heart

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Abstract—In rat heart membranes, the addition of guanine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a stable GTP analogue, did not significantly modify the displacement of [^{3}H]PN 200-110 binding by the 1,4-dihydropyridine (DHP) agonist Bay K 8644 and antagonists, nifedipine and nicardipine. These results are in agreement with some previously reported electrophysiological and pharmacological data, and they suggest that there is no direct involvement of a G protein in the modulation of DHP sensitive Ca channels in cardiac cells.

There is increasing evidence that G proteins modulate the activity of voltage-dependent calcium channels, both indirectly and directly. In neurons, G proteins were shown to constitute critical components in coupling certain receptors, including α_2-adrenergic ($\alpha 2$*), γ-amino butyric acid β (GABA-B), adenosine A_1, to the inhibition of voltage-dependent Ca channels [1, 2].

In addition, in cultured rat dorsal root ganglion neurons, it was reported recently [3] that there is an interaction between G protein activation and the effects of Ca channel ligands on the L-type Ca channels. In the presence of internal guanine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolysable guanine nucleotide analogue, the direct G protein activation enhanced the action of Ca channel ligands at their agonist sites on the channel in its resting state [4]. In these experimental conditions, Ca channel antagonists, including D 600, nifedipine and diltiazem, all produced a rapid and marked potentiation of Ca channel currents, an effect prevented by pertussis toxin (PTX) [3].

The interaction between 1,4-dihydropyridine (DHP) binding sites and G proteins was also characterized by means of binding studies. In synaptic membranes of rat brain cortex, the activation of a G protein induced by the *in vitro* addition of guanyl-5′-yl imidodiphosphate (GMP-PNP), a stable GTP analogue, increased the ability of the DHP agonist, Bay K 8644, to displace [^{3}H]PN 200-110 binding. The *in vivo* treatment with PTX abolished the effect produced by the GTP analogue [5]. All these data suggest that, in neuronal cells, the activation of a G protein coupled to the Ca channels may modify the conformation of DHP binding sites.

In cardiac myocytes, however, conflicting results have been reported. Experiments evidenced that cardiac Ca currents may be stimulated by specific G proteins, activated G_S, involving cytoplasmic second messengers or directly coupling membrane receptors to ion channels, independently of cytoplasmic mediators [6, 7]. In contrast, it was observed that PTX-sensitive G proteins did not influence the response to Ca channel ligands in embryonic chick myocytes [8] and the G_S (G protein stimulatory to adenylyl cyclase) activated by β-adrenergic agonists was not coupled with the Ca channels in frog, rat and guinea-pig heart myocytes [9].

The aim of this study was to investigate whether, as reported in rat cortical membranes [5] the *in vitro* addition of a stable GTP analogue, GTP-γ-S, by activating a G protein, may interact with DHP-sensitive Ca channels and modify the ability of DHP agonist, Bay K 8644 and antagonists, nifedipine and nicardipine to bind to their specific sites labelled with [^{3}H]PN 200-110.

* Abbreviations: GTP-γ-S, guanine 5′-*O*-(3-thiotriphosphate); DHP, 1,4-dihydropyridine; G protein, guanine nucleotide binding protein; $\alpha 2$, α_2-adrenergic; GABA, γ-amino butyric acid; GMP-PNP, guanyl-5′-yl imidodiphosphate; G_S, G protein stimulatory to adenylyl cyclase; PTX, pertussis toxin.

Materials and Methods

Membrane preparation. Male Wistar rats (15 weeks old) were killed by decapitation and their hearts were rapidly removed and perfused with ice-cold 5 mM Tris–HCl (pH 7.4) buffer containing 0.32 M sucrose. The atria were quickly removed by dissection. The ventricular tissues were homogenized with Ultra-Turrax (IKA, Labortechnick, T 25) in 10 vol. of 5 mM Tris–HCl buffer containing 0.32 M sucrose and centrifuged at 1000 *g* (Jouan GR 4.11) for 10 min at 4°. The supernatants were carefully decanted and placed on ice. The pellets were rehomogenized and centrifuged as before. Supernatants were carefully decanted, mixed with previous supernatants and centrifuged at 50,000 *g* (Beckman J-21C) for 10 min at 4°. The supernatants were discarded and the pellets were homogenized in 10 vol. of ice-cold 50 mM Tris–HCl (pH 7.4) buffer and centrifuged at 50,000 *g* for 10 min at 4°. The final pellets were resuspended in ice-cold 50 mM Tris–HCl (pH 7.4) buffer containing 2 mM $CaCl_2$ and used immediately in the binding assays. Protein content of tissue homogenate was determined as described previously [10].

Binding assays. Samples of the membrane preparation containing 0.150–0.200 mg protein were incubated with 0.3 nM [^{3}H]PN 200-110 (Amersham, 71.6 Ci/mM) with or without the presence of other drugs. Incubation was carried out in the dark with a sodium lamp at 25° in 50 mM Tris–HCl buffer (pH 7.4; final volume 1 mL) for 90 min. The reaction was terminated by a rapid filtration under vacuum through GF/C filters (Whatman) which were washed twice with 5 mL of ice-cold buffer. The filters were suspended in 7.5 mL premixed liquid scintillation cocktail (Pico-Fluor TM 15 Packard). Radioactivity was measured in a counter (LKB Wallac 1217) at 37% efficiency. Specific binding was defined as the excess over blanks containing 1 μM nicardipine in absence of GTP-γ-S. Assays were performed in duplicate. The DHPs: Bay K 8644 (Bayer), nicardipine (Bayer) and nifedipine (Sigma) were dissolved in ethanol and GTP-γ-S in distilled water. All the dilutions were performed with distilled water. The alcohol quantity in the final incubation mixtures was 3.4 μmol for each incubations. The K_i values were calculated using the method described previously [11]. Data are expressed as means ± SEM and the Student's *t*-test for unpaired comparison was used.

Results and Discussion

In preliminary experiments, we showed that [^{3}H]PN 200-110 bound in a saturable and reversible manner to a single population of sites. The affinity of specific [^{3}H]PN 200-110

Table 1. Inhibition of [^{3}H]PN 200-110 binding by DHP agonist and antagonists in the absence and presence of GTP-γ-S, in heart membranes of rat

	IC_{50} (nM)					
	Control	GTP-γ-S (μM)				
		1	5	10	50	100
Nicardipine	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	1.4 ± 0.2	1.2 ± 0.2
Nifedipine	2.2 ± 0.2	2.3 ± 0.3	2.0 ± 0.2	2.6 ± 0.3	2.7 ± 0.2	—
Bay K 8644	23 ± 1	17 ± 3	21 ± 2	25 ± 2	20 ± 2	25 ± 2

Membranes were incubated with 0.3 nM [^{3}H]PN 200-110 and different concentrations of compounds. DHP were tested at 7–8 concentrations ranging from 0.1 to 5000 nM.

K_i values were calculated as follows: $K_i = IC_{50}/(1 + c/K_d)$ [11] where K_i = dissociation constant of the competing drug, IC_{50} = concentration of the drug inhibiting 50% of [^{3}H]PN 200-110 binding (obtained from inhibiting curve), c = [^{3}H]PN 200-110 concentration and K_d = dissociation constant of [^{3}H]PN 200-110.

Each value is the mean ± SEM of 4 or 8 (control groups) determinations performed in duplicate.

binding (K_d) was 0.21 ± 0.02 nM and the maximal binding capacity was 120.4 ± 6.9 fmol/mg protein. At 0.2 nM, the specific [^{3}H]PN 200-110 binding defined by 1 μM nicardipine in the absence of GTP-γ-S was 65–80% of the total binding. The three DHP Ca channel ligands completely inhibited specific [^{3}H]PN 200-110 binding. The Hill coefficients obtained from inhibiting curves were not significantly different from unity, which is consistent with a non-cooperative interaction at a homogeneous population of binding sites. In the absence or presence of GTP-γ-S, the order of potency of the compounds was nicardipine > nifedipine > Bay K 8644. For the three compounds, IC_{50} values were not significantly different between control experiments and in the presence of different GTP-γ-S concentrations (1–100 μM). Thus, in heart membranes, GTP-γ-S did not modify the binding of the DHP agonist and antagonists (Table 1).

Recently Yatani *et al.* [6] postulated the existence of a possible direct effect of G proteins on Ca channels in cardiac membranes. In membrane patches excised from guinea-pig cardiac myocytes and bovine cardiac sarcolemmal vesicles incorporated into plasma lipid bilayers, GTP-γ-S prolonged the survival of excised Ca channels and G_S, purified from the plasma membranes of human erythrocytes, also prolonged the survival of Ca channels and stimulated the activity of incorporated channels.

However, results obtained in the present binding study do not support the hypothesis of the existence of G protein interacting with sensitive Ca channels in rat heart membranes. Whatever the concentration of GTP-γ-S, there was no significant modification of the binding of the DHP agonist, Bay K 8644 and antagonists, nifedipine and nicardipine. These binding results are opposite to those reported previously in rat cortical [5] and rabbit skeletal [12] membranes. Indeed, in these preparations, the stable analogues of GTP, GMP-PNP or GTP-γ-S, did not modify the binding of the DHP antagonist, nitrendipine but significantly enhanced the displacement produced by Bay K 8644 and its ability to accelerate the rate of dissociation of [^{3}H]PN 200-110, suggesting the existence of G proteins interacting with DHP binding sites. Thus, the activation of a G protein may modify the conformation of DHP binding sites enhancing the binding of Ca channel agonists in membranes prepared from brain cortex and skeletal muscle: such an effect, however, is not found in the heart. In addition, in rat vascular smooth muscle, GTP-γ-S modulated desmethoxyverapamil binding to Ca channels [12]. These observations afford some arguments to the concept of the organ selectivity of the mechanisms modulating Ca channels. Apart from Yatani and co-workers results presented previously [6, 7], our binding data are in agreement with some electrophysiological and/or pharmacological results obtained in cardiac cells. In aggregates of embryonic chick myocytes, pretreatment with PTX did not affect the increase in contractility produced by Bay K 8644. In addition, by using whole-cell patch techniques, it was shown that (i) the inclusion of GTP-γ-S in the patch pipette did not change the kinetics of the Ca current evoked by depolarization and (ii) the effects of Bay K 8644 and nisoldipine were not modified by GTP-γ-S or when the cells were pretreated with PTX [8]. Furthermore, the absence of a direct coupling of G_S proteins to Ca channels in response to the β-adrenergic agonist was observed in frog, rat and guinea-pig heart myocytes [9]. The discrepancies between these results and those previously reported [6, 7] remain unexplained (species or techniques differences?). Our data, nevertheless, provide some additional evidences to suggest that Ca channel currents in myocytes are not regulated by G proteins in the same way as in nerve, skeletal and smooth muscle cells.

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