

Differential Effects of Pertussis Toxin on the Muscarinic Regulation of Ca²⁺ and K⁺ Currents in Frog Cardiac Myocytes

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ABSTRACT The ability of acetylcholine (ACh) to inhibit β -agonist stimulated calcium current was compared to its ability to activate the inwardly rectifying potassium current $I_{K(ACh)}$ in frog atrial myocytes. As suggested by previous studies, ACh inhibited the calcium current at concentrations ($EC_{50} = 8$ nM) significantly lower than those required for the activation of $I_{K(ACh)}$ ($EC_{50} = 101$ nM). The pharmacological profiles of the two responses suggest that despite the differences in agonist sensitivity, both are mediated by the same (m_2) type of muscarinic receptors. Intracellular application of GDP β S, an inhibitor of G protein function, completely abolished both responses, implying that both actions of ACh are coupled to effectors by G proteins. In contrast, intracellular application of pertussis toxin (PTX) shifted to higher concentrations ($EC_{50} = 170$ nM) but did not abolish inhibition of the calcium current by ACh even though the block of the $I_{K(ACh)}$ response was complete. Increasingly large PTX concentrations and/or prolonged PTX treatments revealed a limiting, PTX-resistant inhibitory component that appears to be mediated by a PTX-insensitive G protein distinct from that mediating $I_{K(ACh)}$. For the PTX-sensitive components, the different agonist dependencies of $I_{K(ACh)}$ activation and calcium current inhibition may imply that different G proteins mediate each response although alternate possibilities involving the same G protein either functionally sequestered and/or differentially affected by interactions with effectors, can not be ruled out.

INTRODUCTION

Acetylcholine alters cardiac function by acting on several cellular regulatory pathways. For ion channels, the most prominent of these include the inhibition of the L-type calcium current induced by β adrenergic agonists and the activation of the

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inwardly rectifying potassium current, $I_{K(ACh)}$.¹ Both of these effects are blocked by atropine and are therefore mediated by activation of muscarinic cholinergic receptors (Giles and Noble, 1976; Breitwieser and Szabo, 1985; Fischmeister and Hartzell, 1986; for recent reviews see Hartzell, 1988; Trautwein and Hescheler, 1990; Szabo and Otero, 1990). In atria, the two pathways coexist, whereas in ventricular tissues $I_{K(ACh)}$ tends to be less prominent or absent (Giles and Imaizumi, 1988). Note that in both tissues there is a small, agonist-independent calcium current that is not affected by ACh (see Trautwein and Hescheler, 1990; Fischmeister and Hartzell, 1986). In order not to confuse this basal component with the L-type I_{Ca} observed as a result of β -adrenergic stimulation, we shall designate the latter as β - I_{Ca} .

$I_{K(ACh)}$ is coupled to muscarinic receptors via the guanine nucleotide binding protein G_K that, upon activation by agonist bound receptor, increases the open probability of the inwardly rectifying $I_{K(ACh)}$ channel through a membrane delimited process (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Brown and Birnbaumer, 1990). β - I_{Ca} results from an increased open probability of Ca^{2+} channels by way of a complex process involving β -adrenergic receptor dependent activation of adenylyl cyclase via the G protein G_s , leading to activation of the cAMP dependent protein kinase-A and phosphorylation of the L-type Ca^{2+} channel or associated protein (Tsien, Bean, Hess, Lansman, Nilius, and Nowycky, 1986; Hescheler, Kameyama, and Trautwein, 1986, for a review, see Trautwein and Hescheler, 1990). Muscarinic inhibition of β - I_{Ca} may affect any one or several of these steps. Previous work suggests that a significant portion of the inhibitory action of ACh is on adenylyl cyclase by way of an inhibitory G protein (Breitwieser and Szabo, 1985; Nakajima, Wu, Irasawa, and Giles, 1990; Parsons, Lagrutta, White, and Hartzell, 1991). Other concomitant mechanisms, however, have not been ruled out (Fischmeister and Hartzell, 1987; Fischmeister and Shrier, 1989). The objective of this work was to examine the mechanisms by which muscarinic receptors modulate different effectors under physiological circumstances using the whole-cell patch clamp technique. In particular, we wanted to determine if the actions of ACh on $I_{K(ACh)}$ and β - I_{Ca} are mediated by the same type of muscarinic receptors, if this action is mediated exclusively via G proteins and if so, whether or not the participating G proteins are identical.

METHODS

Solutions and Drugs

The composition of the solutions given below is in millimolar. Ca^{2+} -free Ringer for the preparation of atrial myocytes: 110 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 10 HEPES, pH 7.2 adjusted with NaOH. Dissociation medium: Ca^{2+} -free Ringer supplemented with 1.2 mg/ml collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.08 mg/ml protease (Type XIV, Sigma Chemical Co., St. Louis, MO). Normal K^+ Ringer solution: 90 NaCl, 2.5 KCl, 5 $MgCl_2$,

¹ A functionally important hyperpolarization-activated muscarinic-inhibited cation current, observed in mammalian atrial myocytes (I_f), has also been reported to have a high, nanomolar sensitivity to ACh (DiFrancesco, Ducouret, and Robinson, 1989). A similar current exists in cells of the frog sinus venosus but not those of the atria (Bois and Lenfant, 1990).

2.5 CaCl_2 , 5×10^{-3} tetrodotoxin, 20 HEPES, pH 7.4 adjusted with NaOH (~ 9 mM). Cs^+ Ringer: 20 CsCl, 85 NaCl, 2.0 MgCl_2 , 2.0 CaCl_2 , 5×10^{-3} tetrodotoxin, 20 HEPES, pH 7.4 adjusted with NaOH. Normal internal solution for the patch pipette: 80 K-aspartate, 30 KCl, 0.5 EGTA, 5.0 HEPES, 2.5 MgATP, 0.2 GTP, pH 7.4 adjusted with KOH. Cs^+ internal solution: 120 CsCl, 0.5 EGTA, 5.0 HEPES, 2.5 MgATP, 0.2 GTP, pH 7.4 with KOH. For experiments with pertussis toxin, the internal solutions were supplemented with 50 μM βNAD .

Pertussis toxin (PTX; 100–400 $\mu\text{g}/\text{ml}$ in PBS) and RR toxin (see below) were a gift from Dr. Erik Hewlett, University of Virginia, Charlottesville, VA. Shortly before use, PTX was activated for 15 min at 35°C in a solution containing 1.0 Na_2ATP , 2.0 Na_2EGTA , 0.5% wt/vol Lubrol PX, 0.03% wt/vol bovine serum albumin, 10 Tris, 20 dithiothreitol, pH 7.5 and the PTX stock diluted twofold. The activated PTX was diluted with internal solution as necessary (usually to 4 $\mu\text{g}/\text{ml}$). In control experiments solutions “activated” without PTX and, in some cases substituting a mutant toxin (RR toxin) unable to ADP ribosylate G proteins (Pizza, Covacci, Bartoloni, Perugini, Nencioni, DeMagistris, Villa, Nucci, Manetti, Bugnoli, Giovannoni, Barbieri, Sato, and Rappuoli, 1989), were used to ascertain that no effects are seen in the absence of ADP ribosylation.

The drugs used in the experiments were (–) isoproterenol (Iso), acetylcholine, atropine, pirenzepine, forskolin, all from Sigma Chemical Co. Forskolin was prepared as a stock solution of 10 mM in anhydrous ethanol; isoproterenol solutions were prepared before each experiment from a stock solution (1 mM) containing 1 mM ascorbic acid. AF-DX 116 was a gift of Boehringer Ingelheim (Reims, France).

Preparations

Bullfrog (*Rana catesbeiana*) atrial cells were isolated as described in Breitwieser and Szabo (1988). Briefly, the frog was killed by decapitation and pithing; the heart was removed and washed in, and then perfused at 30°C for 25 min with Ca^{2+} -free Ringer solution, which had been thoroughly oxygenated by bubbling with 100% O_2 . Subsequently, the heart was perfused for ~ 30 min with dissociation medium. The atrium was removed and suspended in low calcium Ringer (Ca^{2+} -free solution supplemented with 200 μM CaCl_2). The tissue was stored in the refrigerator (4°C) for up to 2 d. Myocytes were obtained by gentle agitation of the tissue in the suspension medium.

Sarcolemma was purified from bullfrog and beef heart according to the procedures of Otero and Szabo (1988) and Jones (1988), respectively. Specific activity of muscarinic cholinergic binding sites (measured as [^3H] quinuclidinyl benzilate binding sites) in sarcolemmal preparations were 6–7 pmol/mg (frog) and 3–4 pmol/mg (beef). Before ADP ribosylation, membranes (3.5–4.2 mg/ml) were diluted with one volume of DNase I (2.5 $\mu\text{g}/\text{ml}$) and Lubrol PX (0.1% wt/vol). Pertussis toxin (170 $\mu\text{g}/\text{ml}$ in PBS) was mixed with an equal volume of 100 mM glycine (pH 8.0 with NaOH), 40 mM DTT and 2% (wt/vol) BSA, and activated for 20 min at 35°C. In controls the toxin was replaced by PBS. ADP-ribosylation of membranes (17.5 and 21 μg protein for beef and frog, respectively) by activated toxin (0.6 μg) was performed in a volume of 60 μl ; the final composition of the assay mixture was: 10 mM thymidine, 1 mM ATP, 1 mM EDTA, 0.1 mM GTP, 20 mM Tris (pH 7.8 with HCl) and 5 μM [^{32}P]NAD (7,500 dpm/pmol). After 2 h at 22°C the reaction was quenched with 120 μl of ice-cold NAD 10 mM (pH 7.5 with Tris) and the samples were centrifuged in an Airfuge (Beckman Instruments, Fullerton, CA) at 197,000 g for 5 min. Pellets were suspended in Laemmli's sample buffer and reduced with 5 mM DTT for 1 h at 50°C, followed by alkylation with 20 mM NEM for 15 min at 22°C. Samples were loaded on 10% SDS-polyacrylamide gels (16 \times 14 cm) and electrophoresed according to Laemmli. The gels were stained with Coomassie Brilliant Blue to verify recoveries, dried and autoradiographed for 24–48 h.

Experimental Arrangements

The whole-cell recording configuration of the tight-seal technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used. Cells were allowed to settle in a recording chamber and superfused with extracellular solution. Patch pipettes were fabricated from square bore borosilicate glass (Glass Co. of America, Millville, NJ) using a Flaming-Brown P 80/PC electrode puller (Sutter Instruments Co., San Rafael, CA) and had resistances between 2 to 5 M Ω when filled with standard intracellular solution. Membrane currents were measured using an Axopatch 200A current to voltage converter (Axon Instruments, Inc., Foster City, CA). Some experiments were performed using custom-built electronics described in Breitwieser and Szabo (1988). Ag/AgCl electrodes were used to establish electrical contact with pipette and bathing solutions. The signal from the current to voltage converter was filtered (5 KHz, 8 dB/octave Bessel), recorded for archival on magnetic media and digitized using a DSP-assisted data acquisition board (DAP 2400, Microstar Laboratories, Bellevue, WA) with 12-bit precision at a rate of 1,000 samples/s and analyzed on-line using software integral to the DAP 2400. Voltage clamp command potentials were also generated by the DAP 2400. Most data were obtained from ionic currents in response to 250-ms depolarizing pulses to -5 mV from a holding potential of -85 mV, applied at a rate of 0.67 Hz. (standard protocol). Initial peak inward currents and steady state pulse-end currents (10 ms average) were calculated for each response. Calcium currents were calculated as peak inward minus steady state current. Muscarinic K⁺ currents were calculated as the change in the pulse-end steady state current after a 1.5-min exposure to extracellular ACh. For some experiments $I_{K(ACh)}$ was measured by clamping the membrane potential to -35 mV and continuously monitoring the membrane current during exposure to extracellular ACh.

Rapid changes of the extracellular solution were obtained by placing the cell near the tube from which solution was perfusing the recording chamber at a velocity of ~ 5 cm/s and switching between different solutions using a rotary valve. This arrangement allowed complete solution changes within 50 ms. Solutions were applied to the interior of the cell via the patch electrode that could be perfused during the experiment using the pressure injection method described elsewhere (Lapointe and Szabo, 1988).

Mean values \pm SEM are given in the text. Curve fitting and statistical analysis were performed using the computer program SigmaPlot (Jandel Scientific, San Rafael, CA). All experiments were carried out at room temperature (21–23°C).

RESULTS

Differential Effects of ACh on I_{Ca} and $I_{K(ACh)}$ in Atrial Myocytes

A review of previous data suggests that acetylcholine inhibits β - I_{Ca} at significantly lower concentrations than those required for eliciting $I_{K(ACh)}$ although direct comparisons on the same preparation and under identical circumstances are usually not available (but see Giles and Noble, 1976; Nargeot, Garnier, and Rougier, 1981). We have examined this issue by determining the dose-effect relationships in frog atrial myocytes where both $I_{K(ACh)}$ and β - I_{Ca} are present.

The dose dependence of $I_{K(ACh)}$ was determined by measuring quasi steady state K⁺ currents induced by 1.5 min. exposure to extracellular ACh using the voltage clamp pulse protocol described in Methods. Care was taken to separate successive agonist applications by at least 3 min so that the cell could recover from any desensitization of the response (Pappano and Mubagwa, 1991). Under these conditions the dose dependence of $I_{K(ACh)}$ was independent of the order in which various ACh concentra-

tions were applied. Typical traces are shown in Fig. 1 *A* as lines connecting individual $I_{K(ACh)}$ determinations made at 1.5-s intervals. Responses normalized to the effect elicited by 10 μ M ACh are plotted as a function of ACh concentration in Fig. 2 (*filled squares*). The solid line was obtained by a nonlinear fit of the Michaelis equation with the maximal response set to unity yielding the half maximal agonist concentration $EC_{50} = 101$ nM.

Corresponding experiments for the inhibition of β - I_{Ca} were carried out in CS^+ containing internal and external solutions in order to eliminate any interference from ACh induced K^+ currents. Isoproterenol (Iso, 2 μ M) was used to elicit a maximal β - I_{Ca} response. After the current stabilized, increasing concentrations of ACh were

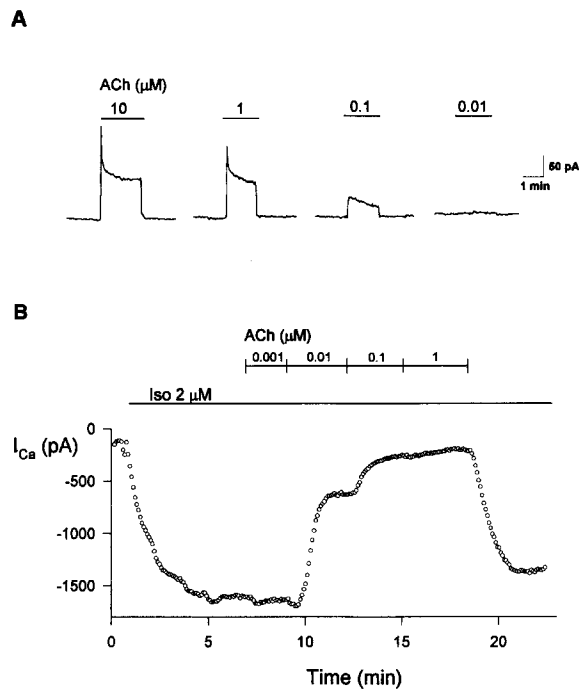


FIGURE 1. Differential agonist sensitivities of $I_{K(ACh)}$ and β - I_{Ca} . (*A*) Muscarinic potassium currents were measured at 1.5-s intervals at the end of 250-ms depolarizing pulses to -5 mV from a holding potential of -85 mV. Representative traces of $I_{K(ACh)}$ are shown for the indicated concentrations of extracellularly applied ACh. Note that individual data points are joined. (*B*) Calcium currents were measured as the peak inward current during a 250-ms depolarizations to -5 mV from a holding potential of -85 mV, and plotted as a function of time. β - I_{Ca} was induced by 2 μ M isoproterenol and the muscarinic inhibition of β - I_{Ca} was assessed by applying ACh at the indicated concentrations in the presence of 2 μ M isoproterenol.

applied to assay the dose dependence of the muscarinic inhibition. Calcium currents were measured as the peak inward current relative to the current measured at the end of 250 ms depolarizations to -5 mV from a holding potential of -85 mV. These transients, obtained at 5.25-s intervals, were plotted as a function of time as shown in Fig. 1 *B* for a typical experiment. Note that at the lowest concentrations (near 1 nM) ACh often produced a slight increase in β - I_{Ca} , observed also in other preparations (Fischmeister and Shrier, 1989), but not in the absence of Iso. Half maximal inhibition was always near 10 nM ACh and was independent of previous ACh applications provided that β - I_{Ca} has been allowed to recover fully. The dose dependence of the inhibitory response is shown in Fig. 2 as filled circles. The dashed

line is a nonlinear fit of data to the Michaelis equation yielding a maximal effect of 99% and a half-maximal inhibitory concentration $EC_{50} = 8.0$ nM. Clearly, even in the same preparation and under the same experimental conditions, ACh is about an order of magnitude more potent in inhibiting β - I_{Ca} than inducing $I_{K(ACh)}$.

Identification of Receptor Types

The difference in the potency of ACh for activation of $I_{K(ACh)}$ compared with the inhibition of β - I_{Ca} raises the possibility that these muscarinic cholinergic effects are mediated via distinct receptor subtypes. This issue was addressed by the use of the subtype specific antagonists pirenzepine and AF-DX 116 (Nathanson, 1987). For $I_{K(ACh)}$ the effects of these antagonists were determined from the magnitude of the outward current at the end of a 1-min application of 1 μ M ACh plus antagonist at a holding potential of -35 mV. The responses were normalized to control (1 μ M ACh alone), obtained at least 3 min preceding the test responses in order to minimize any effects of desensitization. The results are shown in Fig. 3 A. The data were fitted to the Hill equation using nonlinear least squares procedure. With the maximal effect

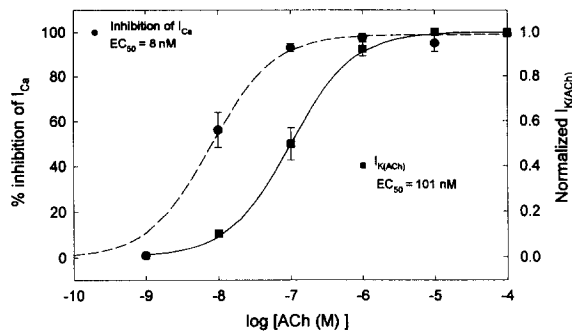


FIGURE 2. Dose-effect relationships for the activation of $I_{K(ACh)}$ and the inhibition of β - I_{Ca} by ACh. The solid lines were drawn to the Michaelis equation, as discussed in the text.

set to 100%, this procedure yielded values of $IC_{50} = 0.49$ μ M and 0.38 μ M for pirenzepine and AF-DX 116 respectively, as well as a Hill coefficient $n_H = 1.0$ in both cases.

The relative effectiveness of the antagonists for relieving the muscarinic inhibition of β - I_{Ca} was assessed by examining the magnitude of β - I_{Ca} that reappeared after application of increasing concentrations of the antagonist together with 1 μ M Iso and 1 μ M ACh. The results of these experiments are shown in Fig. 3 B. The data were fitted to the Hill equation with the maximal effect set to 100%. For pirenzepine, the best fitting parameters were $IC_{50} = 1.7$ μ M and $n_H = 1.7$ while for AF-DX 116 the corresponding parameters were $IC_{50} = 2.3$ μ M and $n_H = 1.44$.

The AF-DX 116/pirenzepine IC_{50} ratios are near unity, 1.3 and 0.74 for $I_{K(ACh)}$ and β - I_{Ca} respectively, suggesting that similar or identical muscarinic receptor subtypes mediate these responses. One can estimate antagonist dissociation constants ($K_{i(antagonist)}$) using the relationship:

$$K_{i(antagonist)} = IC_{50(antagonist)} / (1 + [agonist] / EC_{50(agonist)}). \quad (1)$$

Table I. compares these estimates to corresponding K_i 's reported for a number of muscarinic receptor subtypes.² In contrast to mammalian cardiac mACh receptors (Hammer, Giraldo, Schavi, Monferini, and Ladinsky, 1986; Buckley, Bonner, Buckley, and Brann, 1989), the bullfrog cardiac mACh receptor has high affinity for both pirenzepine and AF-DX 116, in a manner reminiscent of chick heart mACh receptors. Moreover, Table I shows a particularly strong similarity between K_i 's for

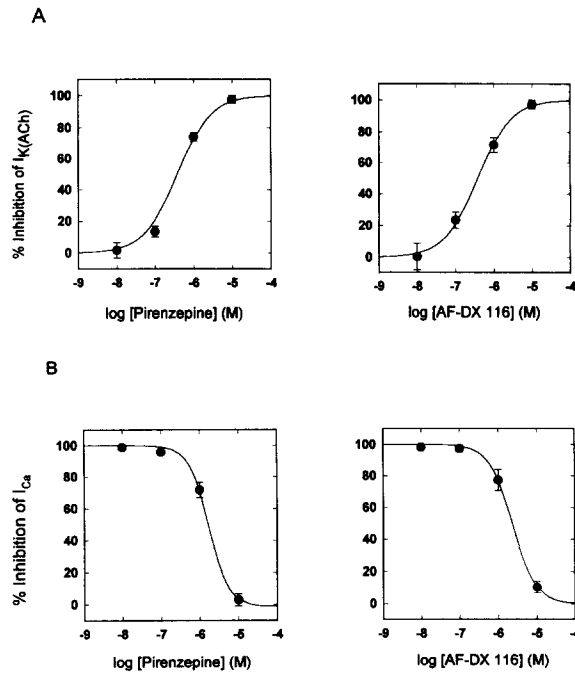


FIGURE 3. Comparison of the effectiveness of the subtype-specific muscarinic antagonists pirenzepine and AF-DX 116 in blocking $I_{K(ACh)}$ and relieving the inhibition of β - I_{Ca} . (A) Antagonist at the indicated concentrations were applied extracellularly together with 1 μ M ACh. The degree of inhibition was calculated as the percent of $I_{K(ACh)}$ remaining after 1 min applications of antagonist containing solutions relative to the corresponding control response observed with ACh alone. Applications were separated by at least 5-min periods of washout with normal Ringers solution in order to minimize possible effects of desensitization. Each data point represents averages of three to seven

measurements. The Hill equation was fitted to the data (solid lines) as discussed in the text. For pirenzepine, $IC_{50} = 0.40 \mu$ M and $n_H = 1$. For AF-DX 116, $IC_{50} = 0.38 \mu$ M and $n_H = 1$. (B) β - I_{Ca} was generated by applying 1 μ M isoproterenol and measured as in Fig. 1. Antagonist effectiveness was assayed by sequential applications of 1 μ M ACh together with antagonist (0.01, 0.1, 1, 10 μ M). Data are plotted as percent of β - I_{Ca} inhibition relative to that produced by 1 μ M ACh in the absence of antagonist. The data were fit to the Hill equation, as described in the text. For pirenzepine, $IC_{50} = 1.7 \mu$ M and $n_H = 1.7$. For AF-DX 116 $IC_{50} = 2.3 \mu$ M and $n_H = 1.4$.

bullfrog atrial mACh receptors and the m_2 mACh receptor subtypes identified in chick heart (Tietje and Nathanson, 1991), suggesting that both the inhibition of β - I_{Ca} and the induction of $I_{K(ACh)}$ are mediated by muscarinic receptors similar to the m_2 subtype present in chick heart.

² Note that Eq. 1 is valid only if the dose-response curve for antagonist has a Hill coefficient of one. Thus, the $K_{D,antagonist}$ values in Table I for β - I_{Ca} may only be approximate.

Differential Effects of Intracellularly Applied Pertussis Toxin

In an effort to distinguish the mechanisms by which muscarinic cholinergic stimulation inhibits β - I_{Ca} and produces $I_{K(ACh)}$, we have examined the influence of pertussis toxin (PTX) on these processes. PTX inhibits G_o/G_i type G proteins by ADP ribosylation of their α subunit (Hazeki and Ui, 1981). PTX was applied intracellularly for two reasons. First, extracellularly applied PTX does not intoxicate frog myocytes unless PTX enters the cells through defects in the cell membrane (Li and Szabo, unpublished observations). Second, intracellularly applied PTX acts rapidly, within minutes of its application. For optimal effects, PTX (usually 4 μ g/ml) was preactivated as described in Methods. The time course with which PTX inhibits $I_{K(ACh)}$ is illustrated in the upper trace of Fig. 4, which plots steady state currents measured at the end of 250-ms depolarizations to -5 mV from a holding potential of -85 mV. $I_{K(ACh)}$ produced by 1 μ M ACh applied at 5-min intervals is seen to decrease in

TABLE I
Comparison of Inhibition Constants for AF-DX 116 and Pirenzepine

System	AF-DX 116	Pirenzepine	Ratio
	<i>nM</i>	<i>nM</i>	
$I_{K(ACh)}$ *	35	45	0.77
β - I_{Ca} *	18	13	1.3
Cm2/CHO [‡]	35	58	0.60
Cm2/Y1 [‡]	18	15	1.2
Cm4/CHO [‡]	199	55	3.6
Cm4/Y1 [‡]	77	38	2.0
Pm1 [§]	2500	18	139
Pm2 [§]	730	660	1.1
Pm3 [§]	2300	180	13
Rm4 [§]	2300	120	19

*Present study, K_j from Eq. 1.

[‡]Tietje and Nathanson, 1991.

[§]Fukuda et al., 1989.

amplitude and vanish after 10 min of intracellular PTX perfusion. The lower trace in Fig. 4 shows I_{Ca} measured and plotted as in Fig. 1. Calcium current was elicited following a complete PTX induced block of $I_{K(ACh)}$ by applying Iso (1 μ M), at ~ 13 min in this experiment. In contrast with the total block of $I_{K(ACh)}$, PTX treatment did not eliminate inhibition of β - I_{Ca} by 1 μ M ACh.

While full PTX action, defined here by a complete loss of muscarinic $I_{K(ACh)}$ response, did not abolish muscarinic inhibition of β - I_{Ca} , it altered markedly the dose dependence for agonist. A typical instance of this is shown in Fig. 5 where the ACh dose dependence of the β - I_{Ca} inhibition was assayed following full PTX action. Rather than decreasing β - I_{Ca} , the inward transients slightly increased following the application of 10 and 100 nM ACh. Inhibition of I_{Ca} becomes evident only at 1 and 10 μ M ACh. This is in contrast with Fig. 1, where in the absence of PTX half maximal inhibition of I_{Ca} was seen for 8 nM ACh.

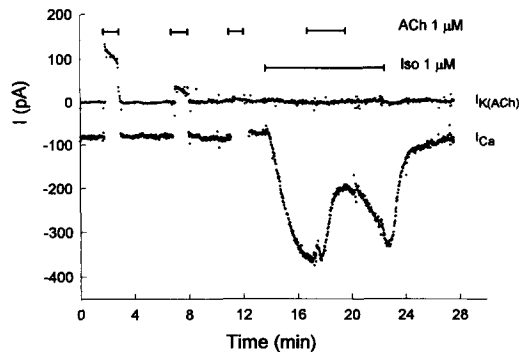


FIGURE 4. Differential effects of PTX treatment on the ability of ACh to induce $I_{K(ACh)}$ and inhibit β - I_{Ca} . $I_{K(ACh)}$ was measured at the end of 250-ms depolarizations to -5 mV from a holding potential of -85 mV, while I_{Ca} was measured as the peak inward current elicited during these same depolarizations. Data are plotted as a function of time ($I_{K(ACh)}$, upper trace; I_{Ca} , lower trace). Note that since $I_{K(ACh)}$ transients contaminate the peak inward current, I_{Ca} was not plotted when a large $I_{K(ACh)}$ was present. Activated PTX ($4 \mu\text{g/ml}$) was included in the patch pipette. Intracellular dialysis began at $t = 0$, the time at which the membrane under the patch pipette was ruptured. As expected, PTX eliminated $I_{K(ACh)}$ but not β - I_{Ca} as shown by responses to extracellular applications of $1 \mu\text{M}$ ACh and $1 \mu\text{M}$ Iso (horizontal bars).

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The ACh concentration dependence of I_{Ca} after maximal PTX treatment is shown in Fig. 6. Data from three to seven experiments of the type shown in Fig. 5 were averaged and plotted against the ACh concentration as filled triangles. The solid line was obtained by a nonlinear curve fitting procedure of the Michaelis equation yielding maximal inhibition of 72% and a half maximal inhibitory concentration of 170 nM. Note that the dose-effect relationship of the PTX-resistant muscarinic inhibition of β - I_{Ca} is not affected by the order of ACh addition and/or previous exposure to ACh, provided that sufficient time is allowed for recovery between additions. As Fig. 6 indicates, the most significant effect of full PTX action is a shift of the dose-effect relationship to higher ACh concentrations; a secondary effect of reducing somewhat the maximal attainable inhibition is also seen.

We have considered the possibility that despite a complete inhibition of the $I_{K(ACh)}$ response by PTX, the remaining muscarinic inhibition of β - I_{Ca} reflects an incomplete action of PTX on its G protein substrates. Thus, we have increased the PTX concentration in the patch pipette and extended the duration of the PTX treatment

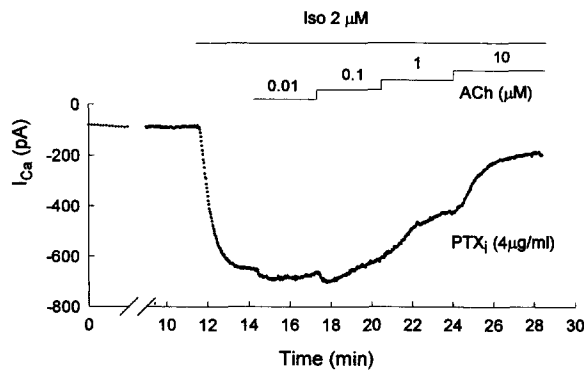


FIGURE 5. Concentration dependence of β - I_{Ca} inhibition by ACh following PTX treatment. The patch pipette contained $4 \mu\text{g/ml}$ PTX; intracellular dialysis started when the whole-cell configuration was attained ($t = 0$). K^+ currents were blocked by using Cs^+ in the internal and external solutions. β - I_{Ca} was measured as in Fig. 1A. Note that half maximal β - I_{Ca} block is not attained until $1 \mu\text{M}$ ACh is applied.

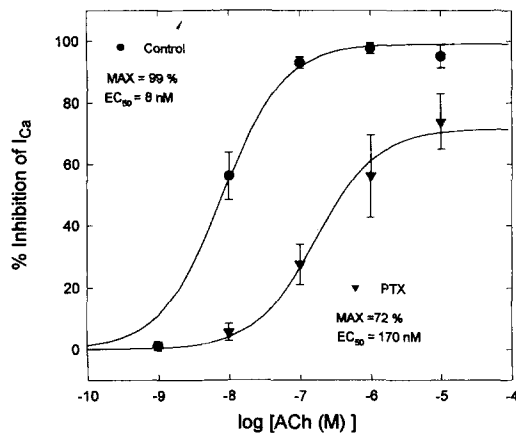


FIGURE 6. Effect of PTX treatment on the ACh concentration dependence of the muscarinic inhibition of β - I_{Ca} . Potassium currents were blocked by Cs^+ in the internal and external solutions. (Filled circles) control inhibitory response obtained in the absence of PTX. (Filled triangles) inhibitory responses following PTX (4 μ g/ml in the patch pipette, at least 10 min intracellular dialysis before application of 2 μ M Iso). Each value represents the average of at least three data points. Solid lines are nonlinear least square fits of the Michaelis equation. For the control response maximal inhibition is 99% and $EC_{50} = 8$ nM; for the PTX maximal inhibition is 72% and $EC_{50} = 170$ nM.

in order to force G protein ADP ribosylation to completion. The results of these experiments are summarized in Table II. Extending the PTX treatment did not abolish the inhibitory response. Rather, the muscarinic block of β - I_{Ca} remained unaltered by further PTX treatment, suggesting that this fraction of the inhibitory response is not mediated by a PTX-sensitive G protein.

Involvement of G Proteins

We have also considered the possibility that part of the inhibitory response (e.g., the PTX resistant part) is not mediated by a G protein. If this is the case then maneuvers that block G protein function should reveal a residual inhibitory response. As we have shown previously, (Li, Otero, and Szabo, unpublished observations; see also Breitwieser and Szabo, 1988; Pfaffinger et al., 1985) $I_{K(ACh)}$ is completely blocked by intracellular application of 1 mM GDP β S. We have used this treatment to block G protein function in general. In the experiment shown in Fig. 7 B, $I_{K(ACh)}$ was abolished by 6 min of GDP β S application in a manner reminiscent of PTX treatment. Forskolin (50 μ M) was applied to stimulate I_{Ca} and ACh (1 μ M) was then applied to test for residual inhibitory response. Fig. 7 B shows the lack of any inhibition of I_{Ca} ; in

TABLE II
Inhibition of I_{Ca} by ACh after Varying Extents of PTX Treatment

PTX treatment	15–20 min	30–40 min	50–60 min
4 μ M/ml PTX	56.1%	60.0%	59.6%
1 μ M ACh	± 23.3 (n = 3)	± 3.1 (n = 2)	± 9.8 (n = 3)
4 μ g/ml PTX	74.0%	—	—
10 μ M ACh	± 15.5 (n = 4)	—	—
20 μ g/ml PTX	70.1%	—	—
10 μ M ACh	± 5.0 (n = 6)	—	—

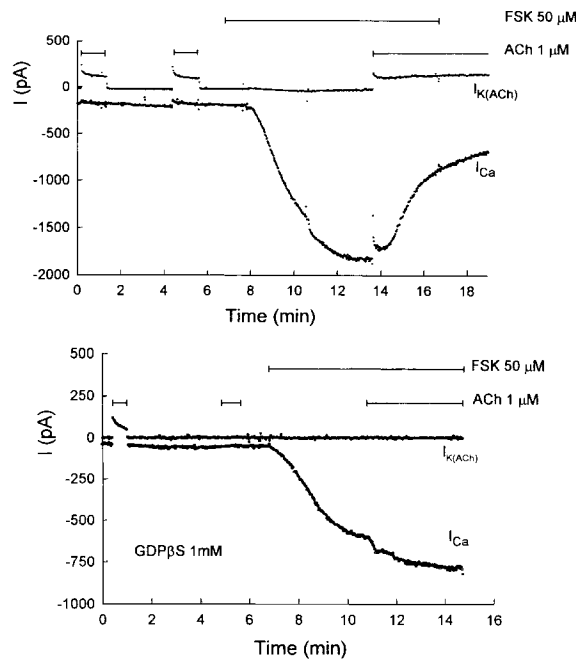


FIGURE 7. The inhibitory response remaining after PTX treatment is mediated by a G protein. (A) The patch pipette was filled with normal internal solution and forskolin (50 μ M) was applied to activate I_{Ca} . As expected, ACh (1 μ M) inhibited this response. (B) The patch pipette was supplemented with 1 mM GDP β S to inhibit G protein function, as verified by a loss of $I_{K(ACh)}$ response. Forskolin (FSK; 50 μ M) was applied to activate I_{Ca} . ACh (1 μ M) had no inhibitory action under these conditions.

fact, one observes a slight enhancement of I_{Ca} . Note that in the absence of intracellular GDP β S one observes a large inhibition of I_{Ca} (Fig. 7A) in agreement with previous reports (Hartzell and Fischmeister, 1987; Fischmeister and Shrier, 1989). The fact that intracellular GDP β S completely abolishes the inhibitory response implies that both the PTX sensitive and the PTX resistant components of the muscarinic inhibition of β - I_{Ca} are mediated by G proteins.

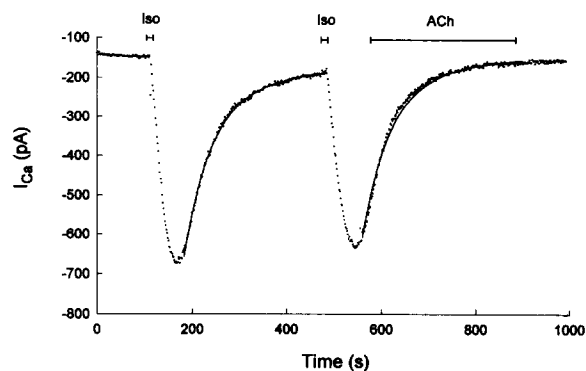


FIGURE 8. Lack of muscarinic effects on the recovery from β -adrenergic activation. Brief applications (5 s) of isoproterenol (1 μ M) were used to induce β - I_{Ca} . After a control response (first transient), a second response was produced and ACh (1 μ M, horizontal bar) was applied during the decaying phase of the response. The time course of the control decay was fitted by an arbitrary function (the sum of two exponentials, as described in the text) and drawn as a solid line. The same function, scaled by a factor of 0.96, was drawn for the decaying phase of the second transient, obtained in the presence of ACh. The close agreement of the data points with the solid line indicates that ACh has little effect on the rate of β - I_{Ca} decay.

Lack of Muscarinic Effects on the Recovery from Beta-Adrenergic Activation

It is possible that part of the muscarinic inhibition of β - I_{Ca} does not result from inhibition of Ca^{2+} channel activation by β -agonist but rather an enhanced rate of recovery of the system from activation, for example due to enhanced cAMP degradation and/or increased phosphoprotein phosphatase activity. In this case application of ACh following a short pulse of Iso, when recovery is in progress, should accelerate the decay of β - I_{Ca} . Fig. 8 shows that contrary to this expectation, the presence of ACh does not alter the decay of β - I_{Ca} . Inward Ca^{2+} transients elicited by depolarizing pulses of -5 mV from a holding potential of -85 mV were measured and plotted as in Fig. 1. The first response shows β - I_{Ca} induced by a 5-s application of $1 \mu\text{M}$ Iso. The decay of β - I_{Ca} could be approximated by a sum of two exponentials and a constant (*solid line*):

$$I = \alpha_0 + \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} \quad (2)$$

with $\alpha_0 = -100$ pA, $\alpha_1 = -436$ pA, $\tau_1 = 60$ s, $\alpha_2 = -121$ pA and $\tau_2 = 936$ s. The second response was also induced by a 5-s application of $1 \mu\text{M}$ Iso, however, $1 \mu\text{M}$ ACh was applied during the recovery phase (*horizontal bar*). The solid curve for the recovery phase was drawn according to Eq. 2 using an offset (α_0) of -100 pA, the same time constants as for the first response, but with amplitudes α_1 and α_2 reduced by the same factor, 0.96. The curve approximates the experimental data closely, indicating that the time course of decay of β - I_{Ca} is not significantly affected by muscarinic stimulation. Note that the same results are obtained when ACh is applied before the control response or when ACh is applied immediately following Iso (data not shown). Together with the observation of the effects of ACh on intracellular cAMP (Fischmeister and Hartzell, 1986; see Trautwein and Hescheler, 1990), these data suggest that ACh has little or no effect on the deactivation of β - I_{Ca} . Thus, the PTX insensitive component of the muscarinic β - I_{Ca} inhibition can not be explained by agonist effects on deactivation processes.

DISCUSSION

Differential Agonist Sensitivities of $I_{K(ACh)}$ and β - I_{Ca} Inhibition

The muscarinic potassium current $I_{K(ACh)}$ can be induced in sino-atrial, atrial and atrio-ventricular tissues, but it is not prominent in mammalian ventricle. β - I_{Ca} is present in both atrial and ventricular tissues, but it has a larger amplitude in the ventricle. For these reasons $I_{K(ACh)}$ has been studied in atrial tissues and β - I_{Ca} mostly in ventricular tissues (Giles and Noble, 1976). Although not performed on the same preparation, these early studies nevertheless tended to suggest that ACh inhibits β - I_{Ca} at concentrations significantly lower than those required to induce $I_{K(ACh)}$. Using the sucrose gap technique, Nargeot et al. (1981) observed differential sensitivities in frog atrial trabeculae, EC_{50} of 80 nM and $1 \mu\text{M}$ for β - I_{Ca} inhibition and $I_{K(ACh)}$, respectively. Here we extended these considerations to isolated bullfrog atrial myocytes using the patch clamp technique. We find a half maximal inhibition of β - I_{Ca} by ACh of 8 nM which is significantly lower than that required to induce $I_{K(ACh)}$, 101

nM. The dose dependence of both effects can be fitted by the Hill equation using a Hill coefficient of one.

It is interesting to note that at nanomolar concentrations ACh appears to stimulate β - I_{Ca} slightly but consistently (see Fig. 1A), an effect that becomes more apparent following PTX treatment, see Fig. 5. It is in fact possible that a rapidly developing, dose dependent potentiation of β - I_{Ca} underlies the apparent lag seen for the inhibition of β - I_{Ca} by ACh. Related observations have been reported by Gallo, Alloatti, Eva, Oberto, and Levi (1993), albeit for much higher concentrations of agonist. It is likely that the ACh-induced increase of β - I_{Ca} is responsible for the lack of inhibitory response seen at 1 nM ACh (Fig. 2; note that the data point falls below the dashed line). This phenomenon may also have physiological implications.

Identity of Receptor Subtypes Mediating $I_{K(ACh)}$ and β - I_{Ca} Inhibition

We have addressed the possibility that $I_{K(ACh)}$ and β - I_{Ca} respond to ACh differently because they are mediated by different types of muscarinic receptors. Mammalian receptor subtypes have been identified pharmacologically based on differences in ligand affinities; pirenzepine, a preferential antagonist of m_1 receptors, and AF-DX 116, a preferential antagonist of m_2 receptors, have been particularly useful in this respect (Hammer et al., 1986; Buckley et al., 1989). We have used these antagonists in an attempt to differentiate and identify the receptors acting on $I_{K(ACh)}$ and β - I_{Ca} in bullfrog. In the presence of 1 μ M ACh the IC_{50} for decreasing $I_{K(ACh)}$ was 0.38 μ M and 0.49 μ M respectively for AF-DX 116 and pirenzepine. For the relief of ACh-induced block of β - I_{Ca} the corresponding IC_{50} 's were 2.3 μ M and 1.7 μ M, Fig. 3. The relative antagonist potencies can be compared for these two actions of ACh by comparing AF-DX 116/pirenzepine IC_{50} ratios which are 0.77 for $I_{K(ACh)}$ and 1.3 for β - I_{Ca} . Considering the range of corresponding relative potencies for the various receptor subtypes, some listed in Table I, these ratios are rather similar, suggesting that muscarinic receptors mediating the stimulation of $I_{K(ACh)}$ and the inhibition of β - I_{Ca} are not distinguishable.

It is also possible to estimate antagonist dissociation constants from the data of Fig. 3 provided that one makes certain assumptions about the underlying processes. For the simplest case, that of competitive binding, K_i 's may be calculated using Eq. 1. This was done for $I_{K(ACh)}$ and β - I_{Ca} using EC_{50} 's for ACh in the absence of antagonist (Fig. 2). The results are shown in Table I, together with K_i 's for selected muscarinic receptor subtypes. These are chick m_2 receptors expressed in Chinese hamster ovary (Cm2/CHO); chick m_2 receptors expressed in mouse Y1 cells (Cm2/Y1); chick m_4 receptors expressed in Chinese hamster ovary (Cm4/CHO); chick m_4 receptors expressed in mouse Y1 cells (Cm4/Y1) (Tietje and Nathanson, 1991); porcine m_1 , m_2 , and m_3 receptors expressed in *Xenopus* oocytes (respectively Pm1, Pm2 and Pm3) and rat m_4 receptors expressed in *Xenopus* oocytes (Rm4) (Fukuda, Kubo, Maeda, Akiba, Hidaeki, Mishina, Higashida, Neher, Marty, and Numa, 1989). Considering K_i 's for mammalian receptor subtypes, typical values of which are shown in the last four rows of Table I, and corresponding data for muscarinic effects on $I_{K(ACh)}$ and β - I_{Ca} in bullfrog atria (first two rows), a lack of pharmacological similarity becomes apparent. For the mammalian receptor subtypes the AF-DX 116/pirenzepine K_i ratios are all much larger than unity except for Pm2. While this would suggest an m_2 -like character

for the frog atrial receptors, the magnitude of the individual K_i 's are at least 10-fold larger. In contrast, chick cardiac receptors (rows 4–6 in Table I) show significant pharmacological similarities to the ones detected in frog. In particular, both the AF-DX 116 K_i 's and the AF-DX 116/pirenzepine affinity ratios observed for the frog are very similar to those seen for chick m_2 receptors. These considerations suggest that the muscarinic receptors that mediate the effects of ACh on $I_{K(ACh)}$ and $\beta-I_{Ca}$ in bullfrog atria are similar to the chick m_2 receptor subtype.

The antagonist dependence of the muscarinic inhibition of $\beta-I_{Ca}$ appears to be steeper than that of $I_{K(ACh)}$ (Fig. 3). While our data do not have sufficient resolution to yield statistically well defined Hill coefficients, these are nevertheless clearly different from unity for the data of Fig. 3 B. This apparent cooperativity may reveal complexities in the system with respect to antagonist binding. An alternate possibility is that at low concentrations, antagonists have the simultaneous action of relieving inhibition of $\beta-I_{Ca}$ and inhibiting the small enhancement of $\beta-I_{Ca}$ by ACh. These effects may combine to give the appearance of a steeper than linear concentration dependence. As we have not found a way to eliminate the small, low concentration enhancement of $\beta-I_{Ca}$ by ACh, we can not rule out this possibility.

Taken together, these data indicate that differences in receptor subtypes can not be invoked to explain the differential sensitivity of $I_{K(ACh)}$ and $\beta-I_{Ca}$ to ACh. Note that AF-DX 116 blocks completely the inhibition of $\beta-I_{Ca}$ by ACh, implying that the response, including any PTX insensitive component, is entirely mediated by muscarinic, Cm_2 -like receptors. It should be noted, however, that certain complexities may exist in the behavior of the system. Thus, for example, when nanomolar ACh concentrations are used, pirenzepine is significantly less potent than AF-DX 116 in reversing the inhibitory effect of ACh on $\beta-I_{Ca}$ (Hanf, Li, Szabo, and Fischmeister, 1993). Further studies will be required to elucidate the basis of these complexities.

Part of the Muscarinic Inhibition of $\beta-I_{Ca}$ Is Not PTX Sensitive

Full inhibition of $I_{K(ACh)}$ implies that G_K is subject to inactivation by ADP ribosylation. In contrast, the muscarinic inhibition of $\beta-I_{Ca}$ was not abolished by PTX although the EC_{50} was shifted to higher concentrations, from 8 nM to 170 nM ACh. It would appear, therefore, that muscarinic inhibition of $\beta-I_{Ca}$ is more complex than the activation of $I_{K(ACh)}$.

A possible reason for a PTX-resistant component is that in the frog G_i/G_o type G proteins are resistant to ADP ribosylation. It has been reported, in fact, that recombinant α_o from the toad *Xenopus* shows reduced PTX sensitivity when compared to its mammalian counterparts (Blitzer, Omri, DeVivo, Carty, Premont, Codina, Birnbaumer, Cotecchia, Carron, Lefkowitz, Landau, and Iyengar, 1993). This conclusion was based on experiments where amphibian and mammalian α_o were translated in vitro using the rabbit reticulocyte system, and subsequently ADP-ribosylated in the presence of presumably mammalian $\beta\gamma$. Because these experimental conditions, tailored to ensure efficient translation and modification of mammalian PTX substrates, are not necessarily optimal for amphibian PTX substrates, we performed experiments to investigate whether ADP ribosylation of frog G_o/G_i -type G proteins is normal and comparable to that seen in mammalian systems. Fig. 9 shows the labeling pattern routinely observed when frog cardiac plasma membranes are ADP-ribosylated

with pertussis toxin; results obtained with beef membranes run simultaneously through the procedure at comparable protein concentrations (21 μ g and 17.5 μ g respectively for frog and beef) are also shown. In frog cardiac sarcolemma, the bands that are specifically labeled by the toxin migrate with apparent molecular weights of 39–42 kD, with the lower M_r band(s) being more intensely labeled. ADP ribosylation of beef membranes gives essentially the same result (lane 3). This indicates that the α -subunits of G_i/G_o proteins in native SL membranes from bullfrog heart are as susceptible to ADP-ribosylation by pertussis toxin as those present in mammalian heart membranes. These data, together with the observation that extended PTX treatments well beyond the times and concentrations required for complete suppression of $I_{K(ACh)}$ could not affect the residual muscarinic β - I_{Ca} inhibitory response, suggests a different molecular mechanism for the latter process.

It should be noted that while a large body of evidence suggests that G proteins couple muscarinic inhibition of β - I_{Ca} , primarily through inhibition of adenylyl cyclase (Breitwieser and Szabo, 1985; Fischmeister and Shrier, 1989; Pappano and Mubagwa, 1991), it is nevertheless possible that other mechanisms, possibly not mediated by G proteins, also contribute to the inhibitory response. Our observation that

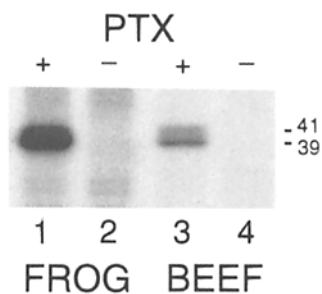


FIGURE 9. ADP ribosylation of G proteins by PTX in cardiac sarcolemma from frog and beef. Autoradiogram of an SDS-polyacrylamide gel is shown. Labeling was performed in the presence (+) or absence (-) of PTX. Apparent molecular weights are marked on the right of the figure. (Lanes 1 and 2) Frog SL; (lanes 3 and 4) bovine SL. The results shown are representative of three experiments with beef membranes and 14 experiments with frog membranes.

intracellular GDP β S completely eliminated the inhibitory effect of ACh, even when the latter was applied at high concentrations suggests, however, that the inhibitory response is mediated entirely by G protein(s).

We find that intracellular GDP β S eliminates $I_{K(ACh)}$ and the muscarinic β - I_{Ca} inhibition with a similar time course so that no residual muscarinic β - I_{Ca} inhibition can be observed once the $I_{K(ACh)}$ response vanishes (see Fig. 7 B). This observation tends to rule out the possibility that a degree of PTX treatment sufficient to eliminate $I_{K(ACh)}$ may leave a residue of unreacted G protein that, because of a strong affinity for the cyclase, may appear as a PTX-insensitive component.

These considerations suggest that muscarinic inhibition of β - I_{Ca} is mediated by at least two G proteins, only one of which is a substrate for ADP ribosylation by PTX. Inhibition of adenylyl cyclase by a PTX-insensitive G protein, G_z , has been reported previously (Wong, Conklin, and Bourne, 1992) and a similar type of G protein may be responsible for the PTX resistant inhibition of β - I_{Ca} observed here. Further experiments using specific G protein reagents will be necessary to establish the identity of the PTX-insensitive G protein involved in the inhibitory process.

Possible Mechanisms

The higher ACh sensitivity of the PTX-dependent β - I_{Ca} inhibitory response relative to that of K^+ channel opening implies significant differences in these signal transduction pathways. The similarities in antagonist binding profiles suggest that the differences are not at the level of the receptor but rather at the level of the G protein/effector system. The most obvious possibility is that these two processes are mediated by different G proteins, that is G_K is not identical to G_i . This must in fact be the case for that portion of β - I_{Ca} which is not sensitive to PTX. In neuronal cells, cyclase activity is inhibited by G_o while the direct activation of a $K(ACh)$ channel is mediated by G_{i3} , that is to say G_K and G_i are different G protein isoforms; this finding is consistent with the participation of two different G proteins in the effects of ACh (see Birnbaumer, 1992). This hypothesis would also explain why desensitization to ACh occurs at a faster rate for the muscarinic activation of K^+ current than for the muscarinic inhibition of β - I_{Ca} (Pappano and Mubagwa, 1991; Honjo, Kodoma, Zang, and Boyett, 1992). An alternative hypothesis, however, is that the same G protein is involved in both effects and the differences arise by way of interactions with the effector either directly through the formation of a preexisting G protein-effector complex or through differential rates of G protein-effector interactions and/or effector-modified rates of G protein deactivation. Kinetic arguments and the lack of cross-talk in agonist binding suggests that, at least in some specific cell types, G proteins are segregated into functional complexes (Graeser and Neubig, 1993). Simple kinetic considerations suggest, however, that our results are also explained by a signal transduction system with mobile components, provided that G protein-effector interactions and G protein deactivation are allowed to be effector dependent. A mechanism of this type would require that inhibition of adenylyl cyclase be more effective and longer lasting than $I_{K(ACh)}$ activation. This may come about as a result of a lower affinity of activated G protein for the K^+ channel relative to cyclase and/or an increased deactivation rate for the G protein- K^+ channel complex. The latter would imply that G protein deactivation may depend on G protein-effector interactions, as seen in other systems (Arshavsky and Bownds, 1992; Pagès, Deterre, and Pfister, 1992; Wong et al., 1992).

Recent experiments suggest that $I_{K(ACh)}$ activation is mediated by G protein $\beta\gamma$ subunits (Logothetis, Kurachi, Galper, Neer, and Clapham, 1987; Murphy, Graber, Garrison, and Szabo, 1993; Wickman, Iniguez-Lluhl, Davenport, Taussig, Krapivinsky, Linder, Gilman, and Clapham, 1994) while adenylyl cyclase inhibition is produced both by G protein α and $\beta\gamma$ subunits (Tang, Iniguez-Lluhl, Mumby, and Gilman, 1992). A mechanism of this type could explain the differences in agonist sensitivities of $I_{K(ACh)}$ and β - I_{Ca} without invoking a second G protein species which would be nevertheless required in order to take into account the PTX-insensitive component we observe. Note, however, that since $I_{K(ACh)}$ activation seems to have no $\beta\gamma$ specificity (Murphy et al., 1993; Wickman et al., 1994) the $\beta\gamma$ subunits produced by activation of PTX insensitive G proteins would be expected to activate $I_{K(ACh)}$. That this is not seen tends to argue against the subunit hypothesis.

During the preparation of this manuscript, nitric oxide has been reported to be an obligatory component of the muscarinic inhibition of β - I_{Ca} in myocytes from the

mammalian sino-atrial node (Han, Shimoni, and Giles, 1994). While we have no indication whether or not this mechanism is present in frog atrial myocytes, our data suggests that if it is present then it must be mediated via a G protein. Further experiments will be required to determine examine the relevance of this mechanism to our observations.

We thank Dr. Erik L. Hewlett for providing us with toxins and Dr. Qing Ye for assistance with some experiments.

This work was partly supported by NIH Grant HL37127 (G. Szabo); an INSERM France-USA exchange program (G. Szabo), a grant from Bayer-Pharma (R. Hanf) and the Fondation pour la Recherche Medicale (R. Fischmeister).

Original version received 18 May 1993 and accepted version received 22 July 1994.

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