Role of GTP-binding Proteins in the Regulation of Mammalian Cardiac Chloride Conductance

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ABSTRACT β-Adrenoceptor agonists activate a time- and voltage-independent Cl^- conductance in mammalian cardiac myocytes. To characterize the cellular signaling pathways underlying its regulation, wide-tipped pipettes fitted with a pipette perfusion device were used to record whole-cell current and to introduce nucleotides to the interior of guinea pig ventricular myocytes. Replacement of pipette GTP with GDPβS prevented activation of the Cl^- conductance by Iso, suggesting a requirement for G protein turnover. With GTP in the pipette, the effect of Iso could be abolished by the β-adrenoceptor antagonist propranolol, and mimicked by histamine or forskolin. These actions of Iso and forskolin are mediated exclusively via cAMP-dependent protein kinase (PKA), because (*a*) maximal activation of the Cl^- conductance by forskolin or pipette cAMP occluded the effect of Iso, and (*b*) switching to pipette solution containing a synthetic peptide inhibitor (PKI) of PKA completely abolished the Cl^- conductance activated by Iso and prevented the action of forskolin, but had no further effect. These results argue against basal activation of the Cl^- conductance, and make it extremely unlikely that the stimulatory G protein, G_s, has any direct, phosphorylation-independent influence. The muscarinic receptor agonists acetylcholine (ACh) and carbachol diminished, in a reversible manner, Cl^- conductance activated by Iso or forskolin, but not that elicited by cAMP. The muscarinic inhibition was abolished by replacing pipette GTP with GDPβS, or by preincubating cells with pertussis toxin (PTX), and was therefore mediated by an inhibitory G protein, presumably G_i, influencing adenylyl cyclase activity. Nonhydrolyzable GTP analogues (GTPγS or GppNHp) applied via the pipette did not themselves activate Cl^- conductance, but rendered Cl^- current activation by brief exposures to Iso or histamine, but not to forskolin, irreversible. The Cl^- conductance persistently activated by Iso was insensitive to propranolol or ACh, but could still be abolished by pipette application of PKI. The data indicate that stimulation of β-adrenergic or histaminergic receptors in the presence of nonhydrolyzable GTP analogues causes persistent activation of G_s and uncouples it from the receptors. We conclude that autonomic regulation of cardiac Cl^- conduc-

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tance reflects accurately the underlying modulation of adenylyl cyclase activity and, hence, that this system is a suitable mammalian model for in situ studies of the interactions between adenylyl cyclase, G_s, G_i, and forskolin.

INTRODUCTION

Agonist activation of β-adrenoceptors leads to modulation of several components of cardiac membrane current (reviewed, e.g., by Hartzell, 1988; Gadsby, 1990) via a coupling of the adrenoceptors to membrane-bound heterotrimeric (comprising α, β, and γ subunits) guanine nucleotide-binding proteins, called G_s (Gilman, 1987), that stimulates adenylyl cyclase and thus raises cellular cAMP concentration. The result is enhanced activity of cAMP-dependent protein kinase (PKA), known to phosphorylate a wide variety of cellular proteins (Cohen, 1988).

This cascade of reactions has been invoked to explain the stimulatory effects of β-adrenoceptor agonists on cardiac dihydropyridine-sensitive Ca^{2+} current (reviewed in Hartzell, 1988), pacemaker current (Chang, Cohen, DiFrancesco, Rosen, and Tromba, 1991; but cf. DiFrancesco and Tortora, 1991), and delayed rectifier K^+ current (Walsh, Begenisich, and Kass, 1988; Yazawa and Kameyama, 1990; Duchatelle-Gourdon, Lagrutta, and Hartzell, 1991), as well as their inhibitory effect on TTX-sensitive Na^+ current (Ono, Kiyosue, and Arita, 1989). However, by analogy with the more direct, membrane-delimited pathway by which another type of G protein, called G_K, coupled to cardiac muscarinic receptors, activates an inwardly rectifying K^+ current (reviewed in Szabo and Otero, 1990; Nanavati, Clapham, Ito, and Kurachi, 1990), several reports have indicated that activated G_s proteins may mediate a phosphorylation-independent influence on dihydropyridine-sensitive Ca^{2+} current (Yatani, Codina, Imoto, Reeves, Birnbaumer, and Brown, 1987; Shuba, Hesslinger, Trautwein, McDonald, and Pelzer, 1990), pacemaker current (Yatani, Okabe, Codina, Birnbaumer, and Brown, 1990), and TTX-sensitive Na^+ current (Schubert, VanDongen, Kirsch, and Brown, 1989). Although the suggestion has been made (e.g., Brown and Birnbaumer, 1988; Yatani and Brown, 1989; Brown, Yatani, VanDongen, Kirsch, Codina, and Birnbaumer, 1990) that the latter, direct pathway provides for an initial rapid response to adrenoceptor activation, while the slower, more persistent, phosphorylation pathway serves to sustain the response, the relative roles of the two pathways remain incompletely understood (Hartzell, Mery, Fischmeister, and Szabo, 1991).

Recently, a novel isoproterenol (Iso)-induced background current was found in guinea pig ventricular myocytes, and its sensitivity to replacement of external Na^+ by TMA^+ prompted the suggestion that it was a new type of Na^+ current (Egan, Noble, Noble, Powell, and Twist, 1987; Egan, Noble, Noble, Powell, Twist, and Yamaoka, 1988). It was subsequently demonstrated, however, that this current is largely carried by Cl^- ions (Harvey and Hume, 1989; Bahinski, Nairn, Greengard, and Gadsby, 1989*b*; Matsuoka, Ehara, and Noma, 1990), and that the effect of Iso could be antagonized by acetylcholine (ACh), and mimicked by exposure to forskolin or by intracellular application of either cAMP or the catalytic subunit of PKA. Moreover, the current elicited by cAMP could be abolished by intracellular application of a specific peptide inhibitor of PKA (Bahinski, Gadsby, Greengard, and Nairn, 1989*a*). Taken together, those studies strongly suggest that PKA-mediated protein phospho-

rylation contributes to the activation of cardiac Cl^- current, but they neither established experimentally the role of G proteins in regulating the Cl^- current nor addressed the question of its possible direct modulation by activated G proteins. Indeed, in none of the above-mentioned investigations of whole-cell Cl^- current was *any* GTP included in the pipette solutions, despite the general presumption that the pipette contents influenced, at least to some extent, the intracellular milieu (e.g., Pusch and Neher, 1988).

In this study we have capitalized on the ability of the intrapipette perfusion technique (Soejima and Noma, 1984), when combined with wide-tipped, whole-cell recording pipettes, to rapidly modify the intracellular solution and so facilitate investigation of the roles played by G proteins in regulating cardiac Cl^- conductance. We confirm that Iso activates the Cl^- conductance through the classical G_s–adenylyl cyclase–PKA pathway, and we show that a pertussis toxin (PTX)–sensitive G protein, presumably G_i, mediates the inhibitory effect of muscarinic agonists. Further, we find no evidence for a more direct phosphorylation-independent pathway in the autonomic activation of cardiac Cl^- current.

METHODS

Preparation of Single Myocytes

Myocytes were isolated from adult guinea pig ventricles as previously described (Isenberg and Klöckner, 1982). Guinea pigs (400–500 g) were fully anesthetized with pentobarbital (~50 mg/kg i.p.). After excision of the heart, the aorta was promptly cannulated and retrograde coronary perfusion at 36°C was begun, initially with oxygenated normal Tyrode's solution, then with oxygenated, nominally Ca-free Tyrode's solution until contraction stopped, and then for 10–15 min with nominally Ca-free solution containing 1 mg/ml (type 1; Sigma Chemical Co., St Louis, MO) or 0.1 mg/ml (Yakult Yakuhin Co., Tokyo, Japan) collagenase. The heart was finally perfused with KB solution (high $[K^+]$, low $[Ca^{2+}]$ and $[Na^+]$ solution; Isenberg and Klöckner, 1982) at room temperature to rinse away the collagenase. The resulting partially digested heart was cut open and papillary muscles and a piece of septal wall were excised, cut into chunks, and then filtered through a nylon mesh. The final myocyte suspension was stored at 4°C in KB solution.

Solutions and Drugs

Normal Tyrode's solution contained (mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES/NaOH (pH 7.4), and 5.5 glucose. The modified Tyrode's solution for superfusion of myocytes contained (mM): 145 NaCl, 1.5 $MgCl_2$, 5 HEPES/NaOH (pH 7.4), 0.5–1 $CdCl_2$, and 5.5 glucose. In some experiments, 0.5–1 mM $BaCl_2$ was added to diminish current flow through inwardly rectifying K^+ channels. The standard pipette solution for intracellular dialysis contained (mM): 85 aspartic acid, 5 pyruvic acid, 10 EGTA, 20 TEACl, 5 $Tris_2$-creatine phosphate, 10 MgATP, 0.1 $Tris_{2.5}$-GTP, 2 $MgCl_2$, 5.5 glucose, and 10 HEPES (pH adjusted to 7.4 with CsOH). Cl^- replaced aspartate in high Cl^- (109 mM) solution.

Nucleotides added to pipette solutions included GTP, or the nonhydrolyzable analogues of GTP, $Li_4GTP\gamma S$ (guanosine 5′-thiotriphosphate) or $Na_2GppNHp$ (5′guanylylimidodiphosphate), or of GDP, $Li_4GDP\beta S$ (guanosine 5′-O-[2-thiodiphosphate]). The synthetic peptide inhibitor (PKI; 5-24-amide) of PKA was prepared as described previously (Cheng, Kemp, Pearson, Smith, Misconi, Van Patten, and Walsh, 1986) and added to pipette solutions at ≤ 100 μM. Agents added to external superfusion solutions included 1 μM Iso, 1 μM forskolin, 5 μM

ACh, 5 μM carbamylcholine (carbachol; CCh), and 10 μM histamine. Stock solutions of Iso (0.8 mM in isotonic Na-lactate buffer), forskolin (10 mM in ethanol), histamine (10 mM), CCh (10 mM), and PTX (50 μg/mg in KB solution) were diluted to the desired final concentration immediately before use. To avoid hydrolysis, ACh stock solution (10 mM) was prepared just before experiment. PTX was dissolved in KB solution at a final concentration for myocyte incubation of 1 or 5 μg/ml, and bovine serum albumin (3 mg/ml) was also added to the incubating solutions for both control and PTX-treated cells.

All agents were purchased from Sigma Chemical Co. except Iso hydrochloride (Winthrop Pharmaceuticals, New York, NY) and forskolin (Calbiochem Corp., La Jolla, CA).

Electrophysiology

Approximately 100 μl of the myocyte suspension was pipetted into the recording chamber on the microscope stage (Diaphot, Nikon Inc., Garden City, NY). Cells were allowed to settle before beginning superfusion with Tyrode's solution. All external solutions were heated to ~36°C in heating coils connected to the two inputs of a two-position valve (Hamilton Co., Reno, NV), one output of which flowed to the chamber via a ~5-cm length of narrow (~0.2 mm i.d.) silastic tubing; the other output flowed to waste. Wide-tipped pipettes (tip diameter ~5 μm, resistance ~1 MΩ) were filled with Tyrode's solution and connected to a device allowing exchange of the pipette solution (Soejima and Noma, 1984). After obtaining gigaohm seals, but before rupturing the membrane patch, the Tyrode's solution was exchanged for standard pipette solution. Once the cell interior was equilibrated (~3 min) with this nominally K^+-free, Na^+-free, and Ca^{2+}-free pipette solution, the extracellular solution was switched to modified Tyrode's solution after setting the holding potential to 0 mV. Under these conditions, K^+ channel currents were eliminated by internal TEA and by omission of K^+ or permeant congeners from both pipette and bath solutions; Na^+ and Ca^{2+} channels were inactivated at 0 mV; Cd^{2+} blocked any residual Ca^{2+} channel current (carried by Ba^{2+}); the nominal absence of internal and external Ca^{2+} prevented Na/Ca exchange current; and Na/K pump current was avoided by the lack of internal Na^+ and external K^+.

Two 3-M KCl half-cells connected the clamp amplifier to the pipette interior and to the chamber to minimize liquid junction potentials. Whole-cell currents were recorded in response to 80-ms voltage pulses to potentials from +100 to −100 mV in 20-mV increments. Current and voltage signals were filtered at 2 kHz, digitized at 8 kHz, stored in an IBM PC computer, and then analyzed with Asyst software (Macmillan Software Co., New York, NY). Steady-state current–voltage (*I–V*) relationships were plotted from current levels averaged over the final 12.5 ms of each pulse. Membrane capacitance was determined by integrating the current transient after a 10-mV step hyperpolarization, and the access resistance was calculated from the decay time constant of that current.

Average data are given as mean ± SEM.

RESULTS

Iso Activates Cl^- Current Solely via β-Adrenoceptors

Internal and external solutions usually contained 24 and 150 mM Cl^-, respectively, so that if the same concentrations obtained at the cell membrane the Cl^- equilibrium potential, E_{Cl}, would have been −47 mV. Hence, at the 0-mV holding potential, activation of Cl^- conductance should have caused an outward current, reflecting net influx of Cl^-. The chart record in Fig. 1 *A* shows that 1 μM Iso (*Iso*) did elicit an outward shift of holding current, which was completely abolished by 2 μM propranolol. The vertical lines on the chart record (*a, b, c*) mark periods of application of brief

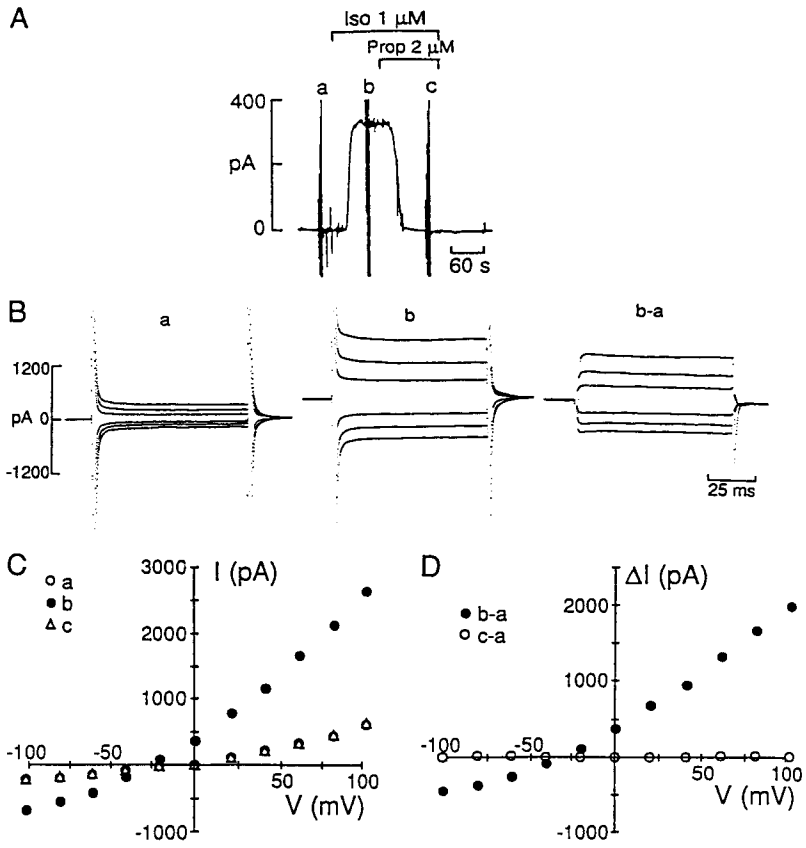

FIGURE 1. Activation of Cl^- conductance by Iso is mediated via β-adrenoceptors. (*A*) Chart record of whole-cell current. Upper bars mark exposures to Iso without, and then with, propranolol (Prop). The vertical lines *a*, *b*, and *c*, indicate application of 80-ms voltage pulses to collect current–voltage (*I–V*) data: holding potential, V_H = 0 mV; cell capacitance, C_m = 227 pF; initial pipette resistance, R_{pip} = 1.4 MΩ; access resistance, R_{acc} = 3.2 MΩ. (*B*) Superimposed sample traces of currents in response to pulses from 0 mV to +60, +40, +20, −20, −40, and −60 mV before (*a*), and during (*b*) the exposure to Iso; the Iso-induced currents (*b* − *a*) were obtained by subtraction of the corresponding digitized records. (*C*) Steady current levels measured over the final 12.5 ms of each trace plotted against pulse potential, before (○) and during (●) exposure to Iso, and after addition of propranolol (△). (*D*) Steady-state difference *I–V* curves obtained by subtraction: ● (*b* − *a*) illustrates Iso-induced current, and ○ (*c* − *a*) demonstrates its complete abolition by propranolol.

voltage pulses, and Fig. 1 *B* shows samples of the resulting current traces obtained before (*a*) and during (*b*) the exposure to Iso. In the absence of Iso, those current traces (Fig. 1 *B*, *a*) and the steady-state, whole-cell *I–V* relationship (Fig. 1 *C*, *a*), indicate that membrane currents were small and roughly time independent, and that they varied practically linearly with membrane potential, confirming that the chosen conditions had eliminated virtually all time- and voltage-dependent components of membrane current.

In the presence of Iso whole-cell currents were large (Fig. 1 *B*, *b* and 1 *C*, *b*), and the Iso-induced currents obtained by subtraction (Fig. 1 *B* and *D*; *b* − *a*) were essentially time independent and showed outward rectification. The interpolated reversal potential of the Iso-activated current (Fig. 1 *D*) was, in this instance, ~ −31 mV, and in 17 cells under these experimental conditions it averaged −34.5 ± 0.9 mV. It is not clear whether this systematic, positive deviation of the reversal potential from the estimated E_{Cl} reflects accumulation of Cl^- just inside the cell, due to the large net Cl^- influx (Fig. 1 *A*), imperfect Cl^- selectivity of the channels, or both.

The complete abolition of the Iso-activated Cl^- current by propranolol, not merely at 0 mV (Fig. 1 *A*; cf. Harvey, Clark, and Hume, 1990) but over the entire voltage range examined (Fig. 1 *D*), confirms that this action of Iso is mediated solely via β-adrenoceptors and rules out, for example, involvement of the novel class of

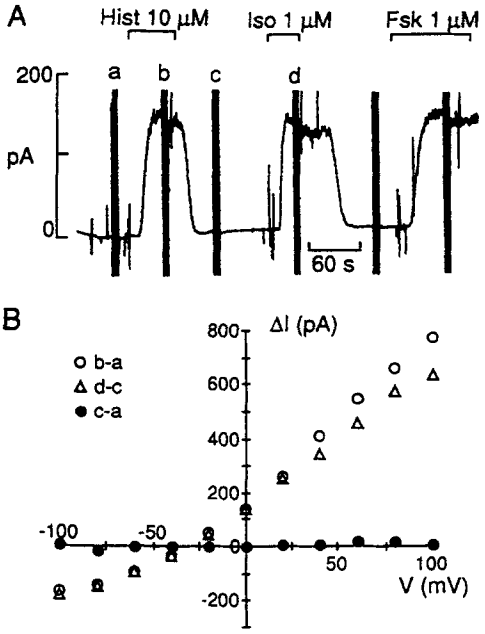

FIGURE 2. Activation of Cl^- conductance by histamine, Iso, and forskolin. (*A*) Chart record of reversible outward current shifts elicited by brief exposures to histamine (Hist), Iso, or forskolin (Fsk) at the 0-mV holding potential: C_m = 198 pF; R_{pip} = 1.0 MΩ; R_{acc} = 3.1 MΩ. (*B*) Steady-state difference *I–V* relationships from *A*, obtained from data identified by letters showing Hist- (*b* − *a*, ○) and Iso-activated (*d* − *c*, △) current; the full reversibility of the effect of histamine is shown by ●, *c* − *a*.

adrenoceptors recently invoked (Tromba and Cohen, 1990) to account for effects of Iso and permeant cAMP analogues on a K^+ current in canine Purkinje myocytes; those effects could not be reversed by either α- or β-adrenoceptor antagonists.

Comparison of Cl^- Currents Elicited by Iso, Histamine, or Forskolin

Since adenylyl cyclase is also activated by histamine via H_2 histaminergic receptors (Hill, 1990), and by forskolin directly (Seamon and Daly, 1986), we compared their effects (cf. Harvey and Hume, 1989, 1990) with that of Iso. As shown in Fig. 2 *A*, 10 μM histamine or 1 μM forskolin elicited outward shifts of holding current similar to that induced by 1 μM Iso (all roughly maximally effective concentrations), all three responses decayed rapidly to baseline (e.g., Fig. 2 *B*, *c* − *a*) after washing away the activator, and the subtracted *I–V* relationships all showed closely similar outward

rectification and reversal potentials (Fig. 2 *B;* see Figs. 4, 6, and 7 for further analysis of the forskolin response).

Not all cells responded to the initial application of Iso (usually ~5–10 min after breaking into the cell). When the pipette solution did not contain GTP (or GDP), 10 of 21 cells (48%) tested with 1 μM Iso showed no change in either holding current or *I–V* relationship, even though in 8 of those 10 cells forskolin elicited a characteristic Cl^- current. In contrast, when 100 μM GTP was included in the pipette solution, only 16 of 95 cells (17%) failed to respond to 1 μM Iso. In 2 of those 16 cells, however, 10 μM histamine did activate the Cl^- current (implying a defect at the level of the β-adrenoceptors), whereas in another two that responded to neither Iso nor forskolin, intrapipette application of cAMP also failed to elicit the Cl^- current (implying a defect closer to the Cl^- channels themselves). To facilitate comparison of the responses between cells, current density was estimated by normalizing the current shift at 0 mV to cell capacitance: excluding those cells that gave no measurable responses to these activators, the average current densities were 1.3 ± 0.5 μA/μF (n = 79) for 1 μM Iso, 0.8 ± 0.1 μA/μF (n = 11) for 10 μM histamine, and 0.8 ± 0.1 μA/μF (n = 68) for 1 μM forskolin. These observations suggest that inclusion of GTP in pipette solutions is essential when wide-tipped pipettes are used to examine responses believed to be mediated via G proteins (Horie, Hwang, and Gadsby, 1992). They also demonstrate qualitative and quantitative variability between cells in their responses to these different activators, presumably reflecting functional differences not only in the density of Cl^- channels but also in the relative densities of the appropriate receptors.

Inhibitory Effect of Muscarinic Receptor Activation

Fig. 3 *A* shows that 5 μM ACh rapidly and reversibly antagonized activation of the Cl^- current by 1 μM Iso (cf. Harvey et al., 1990), the reversibility demonstrating that the effect was attributable to the ACh and not to "rundown" of the channels (e.g., Belles, Malecot, Hescheler, and Trautwein, 1988) or desensitization of the β-adrenoceptors (Hausdorff, Caron, and Lefkowitz, 1990). The difference *I–V* relationships (Fig. 3 *B*) illustrate this inhibitory effect over the full voltage range and show that ACh spared an outwardly rectifying current component with the same reversal potential as the Iso-induced current (Fig. 3 *B*, $c - a$). Although the degree of inhibition was 81% at 0 mV in this example it was seldom as complete, averaging ~50% (Table I) by ACh for the Cl^- current activated by 1 μM Iso, 1 μM forskolin, or 10 μM histamine. A similar extent of inhibition of the Cl^- current activated by 1 μM Iso (Fig. 3 *C*) or 1 μM forskolin (Fig. 9, *A* and *B*) was obtained with another muscarinic agonist, CCh (5 μM). In all of these cases the reversal potential and the voltage dependence of the currents inhibited by ACh or CCh, and of the components remaining, demonstrate that only Cl^- currents were affected.

The latter part of the chart recording in Fig. 3 *C* shows that, in contrast, when the Cl^- current was activated by switching to a pipette solution containing 1 mM cAMP, 5 μM CCh failed to inhibit the current and the spontaneous slow decay proceeded unaltered. To make allowance for this spontaneous decay (roughly linear with time), the equally spaced control sets of *I–V* data, *f* and *h*, were averaged before subtracting

the *I–V* data recorded in the presence of CCh (*g*). The resulting difference *I–V* relationship (△, Fig. 3 *D*) confirms the lack of effect of CCh on the cAMP-activated Cl^- current over the full voltage range. This finding demonstrates that muscarinic inhibition of the Cl^- current occurs at the site of, or prior to, the generation of cAMP. Since Iso, histamine, and forskolin are all known to impinge on adenylyl cyclase, the

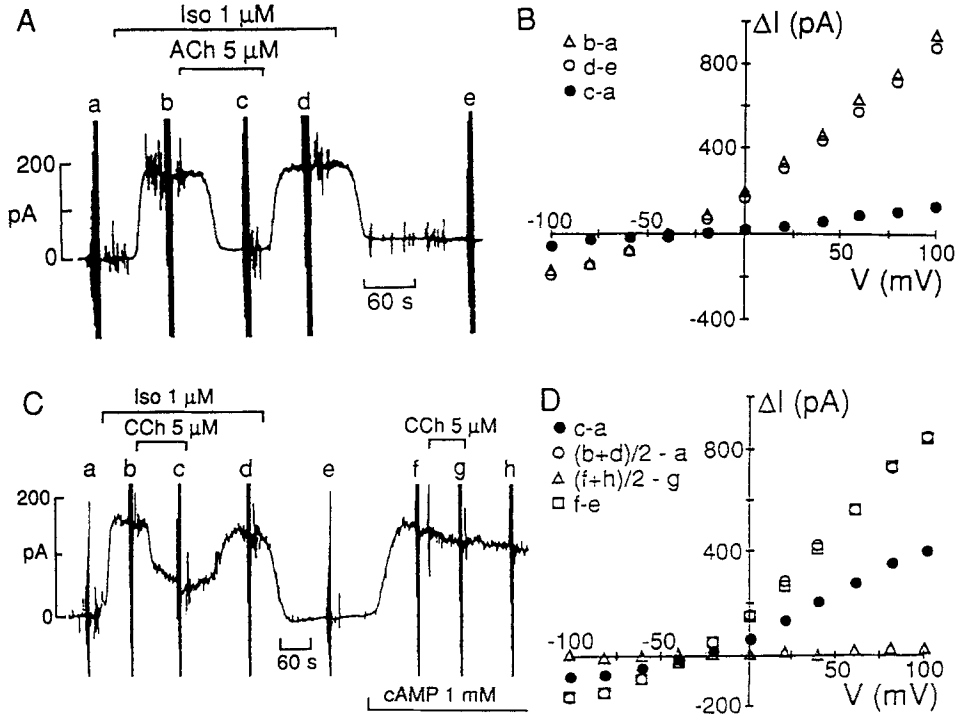

FIGURE 3. Muscarinic inhibition of Iso-induced, but not cAMP-induced, Cl^- conductance. (A) Chart record of whole-cell current at 0 mV: C_m = 154 pF; R_{pip} = 1.0 MΩ; R_{acc} = 2.2 MΩ. (B) Steady-state difference *I–V* relationships showing Iso-activated Cl^- current before (*b* − *a*, △), during (*c* − *a*, ●), and after (*d* − *e*, ○) inhibition by ACh. (*C*) Chart record of holding current at 0 mV showing effects of carbachol (CCh) on Iso-induced current and current activated by intracellular cAMP: C_m = 168 pF; R_{pip} = 1.0 MΩ; R_{acc} = 3.8 MΩ. (*D*) Difference *I–V* curves obtained as indicated: letters correspond to times identified in *C*. To make allowance for slow rundown, average Iso-induced current without CCh was obtained as the difference between *a* and mean of *b* and *d* (○); Iso-induced current during CCh inhibition was obtained as *c* − *a* (●), and the cAMP-induced current without CCh was determined as *f* − *e* (□); the lack of influence of CCh on cAMP-induced current is demonstrated by the null result of subtracting *g* from the average of *f* and *h* (△).

simplest interpretation is that muscarinic receptor activation leads to inhibition of adenylyl cyclase, as already shown for effects on cardiac Ca^{2+} currents (Fischmeister and Hartzell, 1986; Hescheler, Kameyama, and Trautwein, 1986; Parsons, Lagrutta, White, and Hartzell, 1991; cf. Sorota, Tsuji, Tajima, and Pappano, 1985).

Iso Activates Cl^- Current via the PKA Pathway

The above results are consistent with β-adrenoceptor activation leading to stimulation of adenylyl cyclase via the G protein G_s. But because G proteins have been shown to act on ion channels via a membrane-delimited pathway, independent of any cytosolic second messenger, it was important to test whether a similar phosphorylation-independent mechanism might contribute to the activation of cardiac Cl^- current by Iso. If G_s can act directly, then full inhibition of PKA with a synthetic peptide version of the Walsh pseudosubstrate inhibitor, PKI (5-24-amide; Cheng et al., 1986), should neither completely abolish nor fully prevent activation by Iso; moreover, Iso should elicit a further increase in Cl^- conductance when applied during maximal stimulation by forskolin or cAMP.

Fig. 4 shows that after activation of the Cl^- current by 1 μM Iso (Fig. 4 *B*, *b* − *a*, ◇), intrapipette application of 100 μM PKI entirely abolished that current (Fig. 4 *B*,

TABLE I

Percent Inhibition of Cl^- Current by Muscarinic Agonists

Activator	5 μM ACh	5 μM CCh	*n*
1 μM Iso	62 ± 12		5
1 μM Iso		72 ± 14	6
1 μM Fsk	53 ± 12		3
1 μM Fsk		76 ± 7	6
10 μM Hist	54 ± 11		4

After achieving a steady level of Cl^- conductance in the presence of an activator, ACh or CCh was applied (usually for 60–90 s) until a steady degree of inhibition was observed. The percent inhibition represents the magnitude of the inward current shift at 0 mV caused by ACh or CCh, relative to the amplitude of the prior outward current shift caused by the activator, expressed as percentage. Because the inhibition was reversible (e.g., Figs. 3 and 9) and the protocol took only ~5 min to complete, no correction has been made for possible effects of occasional slow rundown, so the percent inhibition given here is an upper limit.

b − *c*, □), presumably because kinase inhibition permitted continuous phosphatase activity to fully dephosphorylate the PKA substrate protein whose phosphorylation is essential for eliciting the Cl^- conductance. That PKI abolishes precisely the Cl^- current activated by Iso, and no more, confirms that the basal level of that phosphoprotein is negligible under our experimental conditions (cf. Bahinski et al., 1989 *b*). The complete abolition by PKI also leaves no room for Iso-induced activation of the Cl^- conductance by any PKA-independent mechanism. Consistent with that conclusion, the difference *I–V* curves in Fig. 4 *B* show that in the presence of PKI there was no effect of either removing (*c* − *d*, ▽) or applying (*e* − *d*, △) Iso. The subsequent lack of response to forskolin, in the presence of PKI (Fig. 4 *A* and Fig. 4 *B*, *g* − *f*, ○), not only confirms that PKA-mediated phosphorylation had indeed been eliminated, but also demonstrates that forskolin has no effect other than activation of Cl^- current (e.g., Figs. 4, 6, and 7) and that this activation occurs strictly

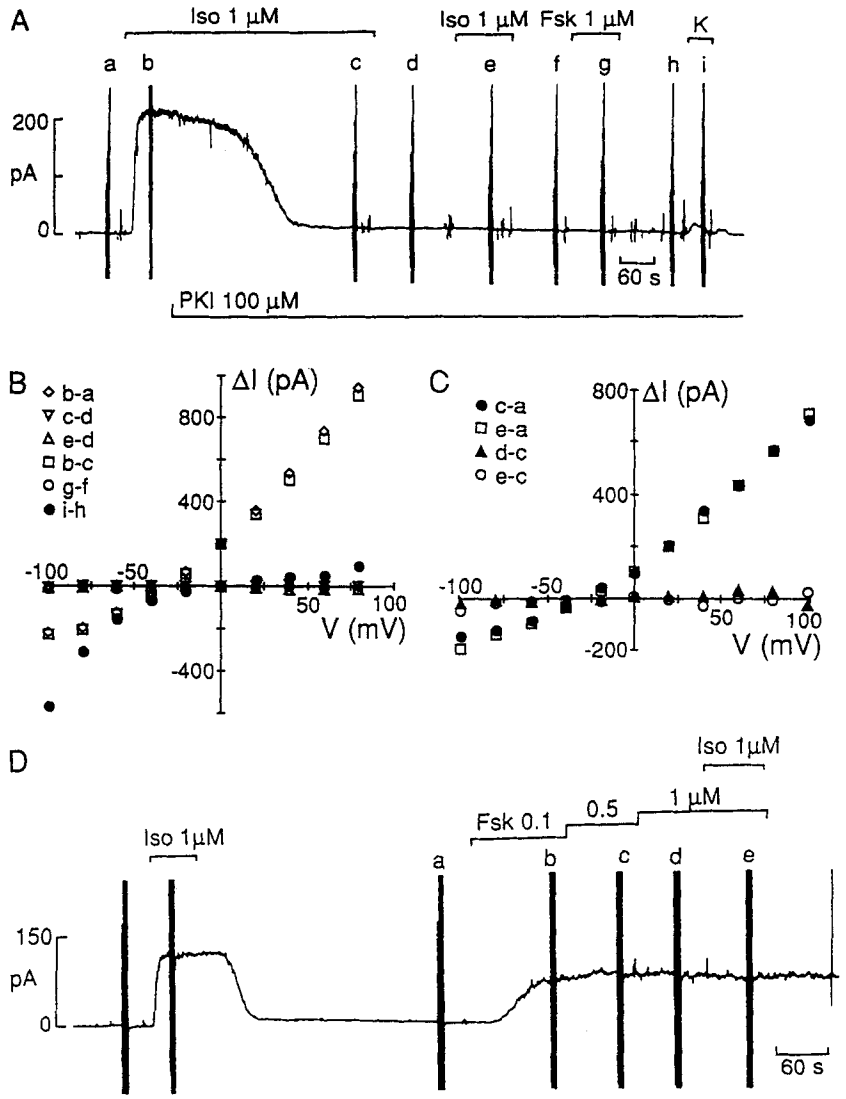

FIGURE 4. (*A*) Chart record of current changes at 0 mV showing complete abolition of Iso-induced Cl^- current by intracellular dialysis with inhibitor peptide, PKI 100 μM (lower bar): C_m = 105 pF; R_{pip} = 0.8 MΩ; R_{acc} = 2.3 MΩ. (*B*) Difference *I–V* relationships obtained as indicated (letters correspond to those in *A*), showing that Iso-activated (*b* − *a*, ◇) and PKI-inhibited (in the presence of Iso, *b* − *c*, □) *I–V*'s are identical and that PKI prevents response to Iso removal (*c* − *d*, ▽) or addition (*e* − *d*, △) as well as response to forskolin (*g* − *f*, ○). Activation of inward rectifier current by exposure to 5 mM K^+ (the external solution was Ba^{2+}-free) is shown by *i* − *h* (●). (*D*) Chart record of current at 0 mV showing lack of response to Iso during maximal activation of Cl^- conductance by forskolin. C_m = 248 pF; R_{pip} = 0.8 MΩ; R_{acc} = 1.5 MΩ. (*C*) Difference *I–V* relationships from *D* (obtained as indicated) showing no effect of raising [Fsk] from 0.5 to 1 μM (*d* − *c*, ▲), nor of adding Iso to the 1 μM Fsk (*e* − *c*, ○); i.e., the Cl^- conductances activated by 0.5 μM Fsk (*c* − *a*, ●) and by 1 μM Fsk plus 1 μM Iso (*e* − *a*, □) were identical. The smaller maximal response to Fsk relative to the initial Iso response probably reflects slow rundown.

through a PKA-dependent pathway. Activation of inwardly rectifying (Fig. 4 *B*, $i - h$, ●), Ba^{2+}-sensitive (not illustrated) current on exposing the cell to 5 mM extracellular K^+ serves simply to confirm the integrity of the cell membrane.

In the experiment of Fig. 4, *C* and *D*, after establishing that the cell responded normally to Iso, forskolin was applied at increasing concentrations. The Cl^- current response to forskolin was already maximal at 0.5 μM, since there was no further effect of raising the forskolin concentration to 1 μM (Fig. 4 *C*, $d - c$, ▲). The smaller maximal Cl^- conductance elicited by forskolin than that initially activated by Iso in this cell is presumably due to a slow rundown process (Horie et al., 1992). Nevertheless, further addition of 1 μM Iso, in the presence of the maximally effective forskolin concentration, produced no further increase in Cl^- conductance (Fig. 4 *C*, $e - c$, ○); i.e., the subtracted *I–V* relationships, $c - a$ (●) and $e - a$ (□), are the same. Similar results were obtained when 1 mM cAMP was introduced into the pipette to maximally activate the Cl^- current, after which exposure to 1 μM Iso was without effect.[1] Thus, Iso has no effect when the cAMP-PKA pathway is either eliminated by PKI or maximally activated by forskolin or cAMP, and so any direct activation of the Cl^- conductance by G_s seems unlikely.

Influence of GTP Analogues on Activation of the Cl^- Current

Because GTP hydrolysis is essential both to terminate signal transduction, and to permit reassociation of the G protein subunits and so restore communication with the adrenoceptor (Gilman, 1987), Cl^- conductance activated by Iso in the presence of a nonhydrolyzable GTP analogue might persist after withdrawing the Iso and be insensitive to β-adrenoceptor antagonists.

Fig. 5 shows that, with GTP in the pipette, a brief exposure to Iso elicited the usual increase in Cl^- conductance (Fig. 5 *B*, $b - a$, ○) which decayed rapidly on washing out the Iso. But after replacing pipette GTP with 100 μM GTPγS, which by itself did not change membrane current (Fig. 5 *B*, $d - c$, ●), another brief exposure to Iso activated an identical Cl^- conductance (Fig. 5 *B*, $e - d$, △) which persisted after withdrawal of the Iso (Fig. 5 *C*, $f - d$, ●) and was insensitive to propranolol (Fig. 5 *C*, $f - g$, △); these latter observations both demonstrate the uncoupling of β-adrenoceptors from G proteins. The persistent Cl^- current was also unaffected by 5 μM ACh (Fig. 5 *C*, $h - i$, ○). However, switching to pipette solution containing 50 μM PKI in addition to the GTPγS abolished the persistent Cl^- conductance (Fig. 5 *D*, $j - k$, ●; $k - d$, △). Under these conditions, the subsequent lack of response to Iso (Fig. 5 *D*, $l - k$, ○) is to be expected on the basis of β-adrenoceptor uncoupling. This *complete* abolition of the Cl^- current by PKI in the presence of GTPγS, despite the fact that the G protein G_s appeared to be irreversibly activated, further argues against any direct activating effect of G_s on the Cl^- channels.

In contrast to this persistence of the Cl^- conductance once it had been activated by Iso and GTPγS, Fig. 6 shows that activation of the Cl^- current by forskolin remained fully reversible during pipette perfusion with GTPγS, indicating that stimulation of adenylyl cyclase by forskolin does not require activation of a G protein.

When 100 μM GppNHp was used instead of GTPγS, Iso-induced Cl^- current also persisted after washout of the Iso. Results using histamine as an agonist to

[1] Horie, M., T.-C. Hwang, A. C. Nairn, and D. C. Gadsby, manuscript in preparation.

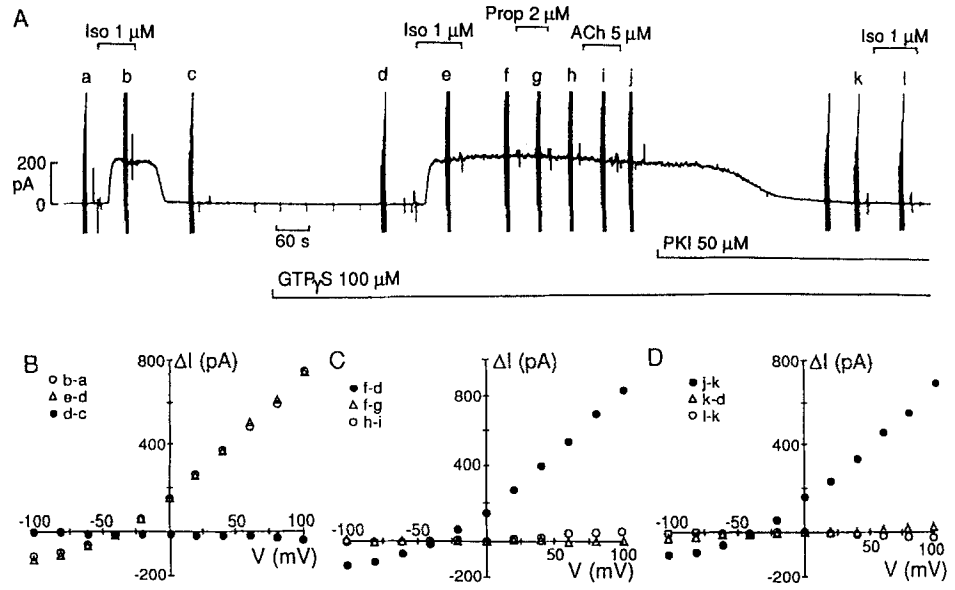

FIGURE 5. Influence of the nonhydrolyzable GTP analogue, GTPγS, on activation and inhibition of the Cl^- conductance. (*A*) Chart record of current at 0 mV. The lower bars show when GTPγS was substituted for pipette GTP, and when PKI was added to the GTPγS: C_m = 176 pF; R_{pip} = 0.7 MΩ; R_{acc} = 2.7 MΩ. (*B*, *C*, and *D*) Difference *I–V* relationships obtained by subtraction, as indicated (the letters correspond to those in *A*). Iso-activated Cl^- conductance in the presence of pipette GTP ($b - a$, ○) is identical to that in the presence of GTPγS ($e - d$, △), and GTPγS itself had no effect ($d - c$, ●). The Cl^- current persisted after washout of Iso ($C, f - d$, ●), and then propranolol ($C, f - g$, △) and ACh ($C, h - i$, ○) were without effect. The Cl^- current abolished by internal PKI ($D, j - k$, ●) displayed similar outward rectification, reversal potential, and magnitude to that initially activated by Iso. The negligible Iso-activated current, ($D, l - k$, ○), confirms the effectiveness of the PKI, and the null *I–V* relationship, $k - d$ (*D*, △), demonstrates both that PKI completely abolished Iso-activated Cl^- conductance in the presence of GTPγS, and that any PKI-insensitive conductance activated by GTPγS was insignificant.

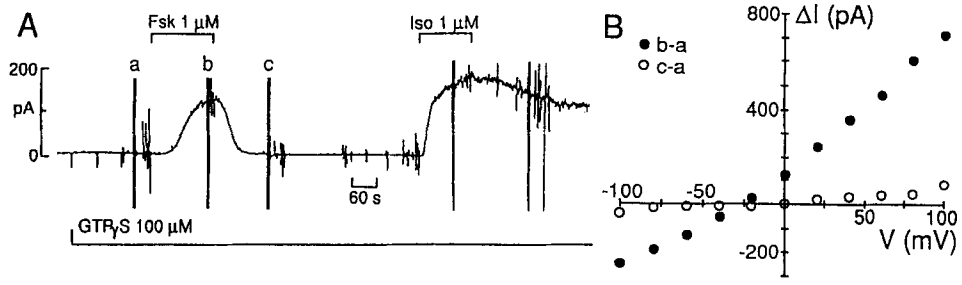

FIGURE 6. Reversible activation of Cl^- conductance by forskolin in the presence of pipette GTPγS. (*A*) Chart record of current at 0 mV. GTPγS was substituted for pipette GTP as indicated by the lower bar: C_m = 56 pF; R_{pip} = 1.1 MΩ; R_{acc} = 5.0 MΩ. (*B*) Steady-state difference *I–V* curves showing characteristic Fsk-induced current ($b - a$, ●) and its reversibility ($c - a$, ○) on washout of forskolin, despite the persistence of Cl^- current subsequently activated by a brief exposure to Iso.

irreversibly activate G_s in the presence of pipette GTPγS (cf. Fig. 8, below) corroborated those described here with Iso.

GDPβS is a nonhydrolyzable GDP analogue that competes with GTP for the nucleotide binding site on G proteins but does not activate them (Eckstein, Cassel, Levkovitz, Lowe, and Selinger, 1979). Fig. 7 shows that after the normal, reversible activation of Cl^- current by a brief exposure to Iso during pipette perfusion with GTP, switching to a pipette solution in which the GTP had been replaced by 1 mM GDPβS prevented, within a minute or two, any further response to Iso (Fig. 7 *B*, *d* − *c*, ▲), although 1 μM forskolin was still able to elicit a full (and fully reversible) response. This result demonstrates that activation of the Cl^- conductance by Iso is strictly dependent on G protein turnover, whereas activation by forskolin is not.

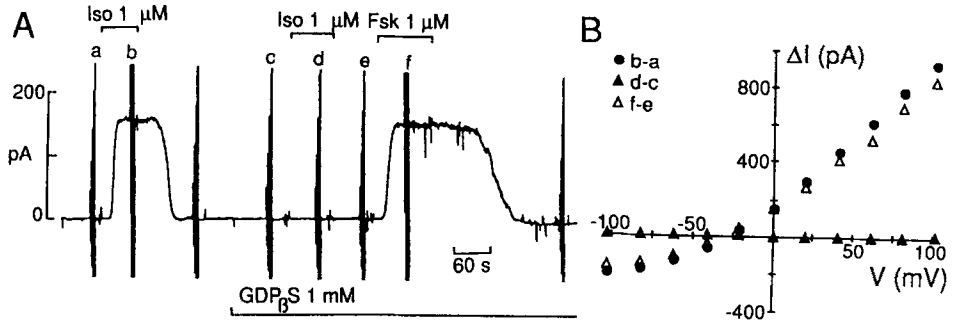

FIGURE 7. Abolition of Cl^- current response to Iso but not to forskolin after replacing 100 μM pipette GTP with 1 mM GDPβS (lower bar). (*A*) Chart record of current at 0 mV: C_m = 181 pF; R_{pip} = 0.5 MΩ; R_{acc} = 1.5 MΩ. (*B*) Steady-state difference *I–V* relationships obtained as indicated, showing that Iso activated Cl^- conductance in the presence of GTP (*b* − *a*, ●) but not in the presence of GDPβS (*d* − *c*, ▲) when Fsk could still activate Cl^- current (*f* − *e*, △).

$[Cl^-]$ Dependence of the Persistently Activated Conductance

The use of hydrolysis-resistant GTP analogues to persistently activate the Cl^- conductance permits characterization of some of its properties free from complications such as receptor desensitization. For example, Fig. 8 shows the effect of changing pipette $[Cl^-]$. A brief exposure to histamine, in the presence of 150 mM external Cl^- and pipette solution containing GTPγS and 109 mM Cl^-, resulted in persistent activation (Fig. 8 *C*, *b* − *c*, ○) of a large conductance with a roughly linear *I–V* relationship (Fig. 8 *C*, *b* − *a*, ●; cf. Bahinski et al., 1989*b*) and an interpolated reversal potential only a few millivolts negative to the 0-mV holding potential (and close to the estimated E_{Cl} of −9 mV). For that reason, histamine caused only a relatively small shift of holding current (Fig. 8 *A*). Lowering the pipette $[Cl^-]$ to 24 mM then caused a large outward shift of holding current (Fig. 8 *A*), although Fig. 8, *B* and *D*, shows that the shift was immeasurably small at the largest positive potentials and greatest at the most negative potentials, as expected for a reduction of inward current due to decreased Cl^- efflux.

The difference current, ΔI_{Cl} (Fig. 8 *D*, *c* − *d*, ●), is analyzed further assuming (*i*) that the switch from 109 to 24 mM pipette $[Cl^-]$ resulted in the same change ($\Delta[Cl^-]_i$) in intracellular $[Cl^-]$, (*ii*) that over the ~4-min period required to obtain the data

only Cl^- efflux through PKA-regulated Cl^- conductance was altered, and (*iii*) that this Cl^- current can be described by the constant field equation. The curve in Fig. 8 *D* shows a least-squares fit to the data of

$$\Delta I_{Cl} = P_{Cl} \frac{VF^2}{RT} \cdot \frac{\Delta[Cl]_i}{[1 - \exp(VF/RT)]}$$

from which the Cl^- permeability, P_{Cl}, is estimated to be $\sim 7 \times 10^{-8}$ cm·s^{-1} if specific membrane capacitance is taken as 1 μF·cm^{-2}.

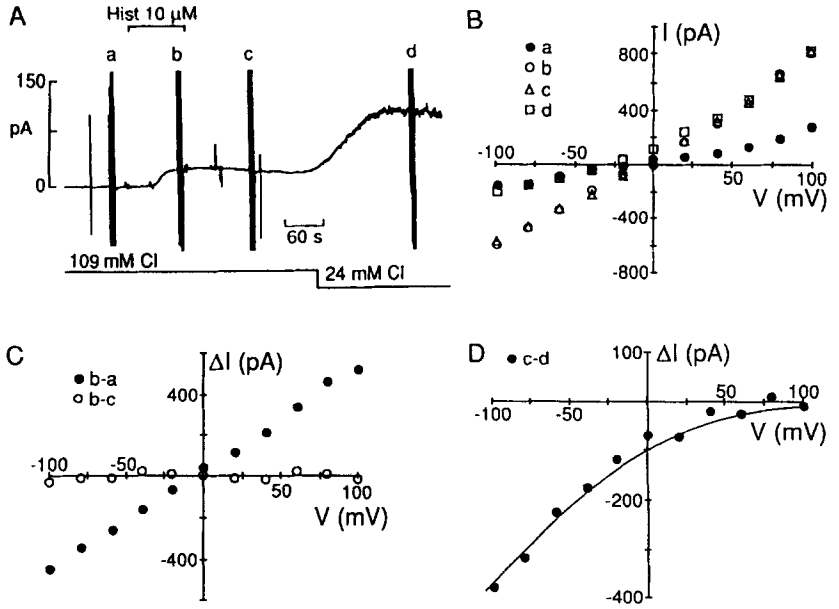

FIGURE 8. [Cl^-] sensitivity of persistent current activated by histamine in the presence of 100 μM pipette GTPγS. (A) Chart record of current at 0 mV; C_m = 171 pF; R_{pip} = 0.8 MΩ; R_{acc} = 2.0 MΩ. After a brief exposure to histamine, the pipette [Cl^-] was lowered from 109 to 24 mM (lower line). (*B*) Steady-state whole-cell *I–V* relationships at 109 mM pipette [Cl^-], before (*a*, ●), during (*b*, ○), and after (*c*, △) exposure to histamine, and after (*d*, ○) lowering pipette [Cl^-]. (C) Difference *I–V* curves showing a relatively linear histamine-activated *I–V* relationship (*b* − *a*, ●) with high intra- and extracellular [Cl] and lack of effect of washing out histamine (*b* − *c*, ○). (*D*) Difference *I–V* relationship (*c* − *d*, ●) showing intracellular [Cl^-]-sensitive current; the smooth curve shows a least-squares fit to the constant field equation (see text).

Persistent activation of the Cl^- conductance in this manner should facilitate systematic testing of effects on the *I–V* relationship of anion substitutes, Cl^- channel blockers, and other possible modulatory agents such as Na^+ ions (cf. Harvey, Jurevicius, and Hume, 1991).

Muscarinic Inhibition of the Cl^- Current Involves a PTX-sensitive G Protein

Hydrolysis-resistant guanine nucleotide analogues also provide a means of investigating the role of G proteins in inhibition of activated Cl^- conductance. Fig. 9 *A*

illustrates the effect of replacing pipette GTP with GDPβS on the muscarinic inhibition of forskolin-activated Cl^- current. Forskolin was chosen instead of Iso because activation of the Cl^- current by forskolin is independent of G protein turnover (Fig. 7, above). With 100 μM GTP in the pipette, a brief exposure to 5 μM CCh caused reversible inhibition of the Cl^- current induced by 1 μM forskolin; the CCh-inhibited *I–V* relationship (Fig. 9 *B*, *a* − *b*, ●) displays the characteristic outwardly rectifying shape and usual reversal potential. However, within 3 min of

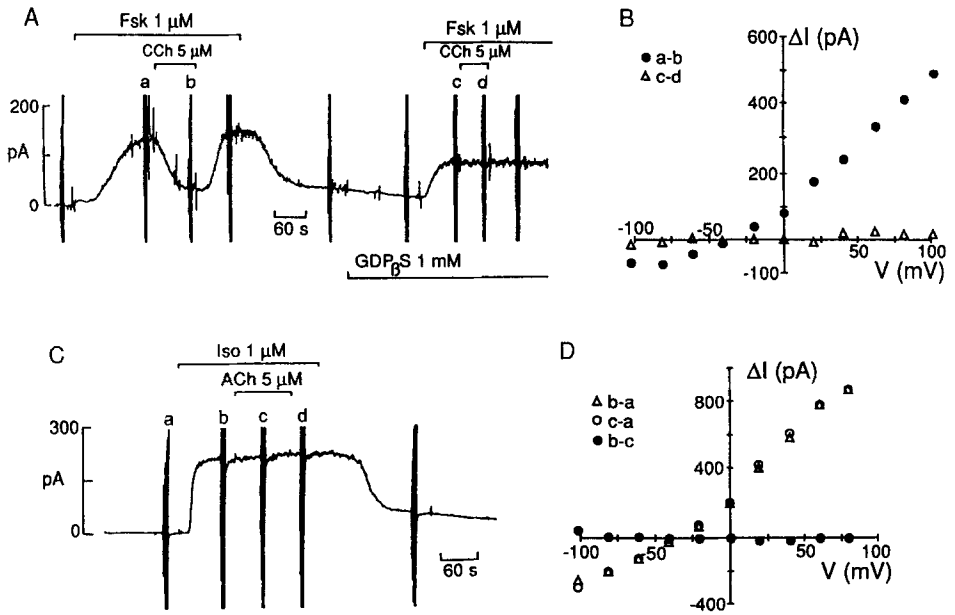

FIGURE 9. Muscarinic inhibition of Cl^- conductance is mediated by a PTX-sensitive G protein. (*A*) Chart record of current at 0 mV: C_m = 110 pF; R_{pip} = 0.9 MΩ; R_{acc} = 1.9 MΩ. (*B*) Steady-state difference *I–V* curves: the component of forskolin-induced current inhibited by CCh in the presence of pipette GTP (*a* − *b*, ●) displayed the outward rectification and reversal potential characteristic of Cl^- current, but with GDPβS in the pipette CCh had no effect (*c* − *d*, △). (*C*) Chart record of current changes, at 0 mV, caused by Iso and ACh in a cell that had been preincubated with PTX (5 μg/ml) for 1 h at 32°C: C_m = 166 pF; R_{pip} = 0.7 MΩ; R_{acc} = 2.1 MΩ. (*D*) Steady-state difference *I–V* relationships showing that the Iso-induced Cl^- current was the same in the absence (*b* −*a*, △) as in the presence (*c* − *a*, ○) of ACh; i.e., ACh had no effect (*b* − *c*, ●).

replacing pipette GTP with 1 mM GDPβS, CCh failed to affect the forskolin-activated Cl^- current (Fig. 9 *B*, *c* − *d*, △), confirming the involvement of a G protein in the muscarinic inhibition.

Fig. 9 *C* demonstrates that the G protein involved is sensitive to PTX. The cell in this experiment had been incubated at 32°C for 1 h in KB solution containing 5 μg/ml PTX plus 3 mg/ml albumin. This procedure had no effect on the Iso-induced Cl^- current (Fig. 9 *D*, *b* − *a*, △), but completely prevented inhibition of that current

by 5 μM ACh (Fig. 9 *D*, *b* − *c*, ●). In three cells treated with 1–5 μg/ml PTX in this way, neither 5 μM ACh ($n = 2$) nor 5 μM CCh ($n = 1$) caused any detectable inhibition of the Iso-induced Cl^- current, whereas in five cells incubated with albumin alone, but under otherwise identical conditions, the inhibition of Iso-induced Cl^- current by 5 μM ACh averaged 57 ± 11%, similar to the 62 ± 12% inhibition observed in five untreated cells. In a further four cells treated with 1 μg/ml PTX at 30°C for 1 h, 10 μM CCh caused no inhibition of the Cl^- conductance elicited by 1 μM forskolin.

DISCUSSION

Several conclusions can be drawn from these results: (*a*) activation of cardiac Cl^- conductance by the β-adrenoceptor agonist Iso, or by histamine, is mediated by a G protein and involves only the adenylyl cyclase–PKA pathway, whereas (*b*) activation of the Cl^- conductance by forskolin occurs *exclusively* via the same adenylyl cyclase–PKA pathway, but does *not* depend upon activity of a G protein; (*c*) it is extremely unlikely that any component of the agonist-elicited Cl^- conductance is activated via a direct, phosphorylation-independent pathway; and (*d*) muscarinic inhibition of the agonist-induced Cl^- conductance occurs at the level of the adenylyl cyclase and is mediated by a PTX-sensitive G protein.

G_s Mediates Activation of Cl^- Current by Iso or Histamine

Occupied β-adrenoceptors cause the G protein, G_s, to release GDP, bind GTP, and dissociate into a βγ subunit and an α-GTP subunit which stimulates adenylyl cyclase; the ensuing rise in cellular [cAMP] activates PKA (Gilman, 1987). Our previous reports, as well those of others (reviewed by Hume and Harvey, 1991), have shown that PKA-mediated phosphorylation leads to activation of cardiac Cl^- current, presumably by phosphorylation of the anion-selective channels identified by Ehara and Ishihara (1990). Our results presented here establish the requirement of G protein activation for Iso and histamine, but not forskolin, to elicit the Cl^- current: (*a*) replacing cellular GTP with GDPβS completely prevented Iso-mediated, but did not alter forskolin-mediated, activation of Cl^- current (Fig. 7); (*b*) on replacing cellular GTP with GTPγS, Iso and histamine caused persistent activation of the Cl^- current (Figs. 5 and 8), whereas the effect of forskolin remained fully reversible (Fig. 6). The occlusion of the Iso response by a maximal concentration of forskolin (Fig. 4) or cAMP,[1] the closely similar magnitudes of the Cl^- currents activated by high concentrations of Iso, histamine, forskolin, or cAMP (Figs. 2, 3, and 7), the sensitivity of the effects of Iso (Fig. 3), histamine (Table I), and forskolin (Fig. 9), but not of cAMP (Fig. 3), to muscarinic agonists, and the abolition of the effects of Iso (Fig. 4), forskolin (Fig. 4), or cAMP (Bahinski et al., 1989*b*) by PKI, all argue that the pathways activated by Iso, histamine, and forskolin converge at the level of adenylyl cyclase and, hence, that G_s is the G protein mediating the stimulatory effects of both Iso and histamine.

In this scheme, the persistent activation of Cl^- current by a brief exposure to Iso in the presence of GTPγS (or GppNHp) is due to perpetually active GTPγS-bound (or GppNHp-bound) α subunits of G_s. Reassociation of α_s subunits with βγ complexes is

prevented and the adrenoceptors are effectively uncoupled from the G_s proteins, accounting for the insensitivity of persistent Cl^- current to the β-adrenoceptor blocker propranolol (Fig. 5; contrast with Fig. 1). Breitwieser and Szabo (1985) observed similarly persistent, G_s-mediated stimulation of Ca^{2+} current in amphibian myocytes in response to a pulse of Iso in the presence of pipette GppNHp, although GppNHp has also been reported to inhibit Iso stimulation of Ca^{2+} current (see discussion in Parsons et al., 1991). However, an alternative explanation for the persistence in Fig. 5 should be considered: transphosphorylation from GTPγS by the enzyme nucleotide diphosphate kinase (NDPK) could generate ATPγS (cf. Otero, Breitwieser, and Szabo, 1988), and PKA could then transfer the thiophosphate from ATPγS to the Cl^- channel protein, yielding a relatively long-lived phosphoprotein (cf. Kameyama, Hofmann, and Trautwein, 1985; Hescheler, Kameyama, Trautwein, Mieskes, and Soling, 1987; Harvey et al., 1991). However, this possibility is unlikely for several reasons: (*a*) persistent activation of almost maximal Cl^- conductance could occur within 3 or 4 min of introducing GTPγS (Fig. 5), probably too short a time for transfer of sufficient thiophosphate from GTPγS to the channel protein, via ATPγS, to cause activation of such a large conductance, considering competition between the maximum possible [ATPγS], 0.1 mM, and the 10 mM ATP in the pipette solution; (*b*) the persistent Cl^- conductance (e.g., Figs. 5 and 8) could reflect long-lived phosphoprotein only if the dephosphorylation rate were extremely low, which cannot be the case because on introducing PKI the conductance decayed (due to dephosphorylation) at the *same rate* in the presence of GTPγS (Fig. 5) as in the presence of GTP (Fig. 4); (*c*) similarly, if transphosphorylation via ATPγS were significant, forskolin should also yield long-lived phosphoprotein in the presence of GTPγS, whereas, on the contrary, Fig. 6 shows that the forskolin response is fully reversible, at the normal rate, in GTPγS; (*d*) on the other hand, deliberate introduction of 2–5 mM ATPγS can indeed lead to a larger amplitude of activated Cl^- conductance and a slowed, incomplete conductance decline on withdrawal of the activator,[1] consistent with the formation of a phosphoprotein more resistant than normal to endogenous phosphatase activity; (*e*) finally, we found persistent activation of Cl^- conductance in the presence of GppNHp, which is not a substrate for NDPK (Otero et al., 1988).

Lack of Direct G Protein Activation of Cl^- Conductance

Although our results clearly demonstrate that the response to Iso *requires* G protein activation (see above) they provide no evidence, and indeed leave little room, for any contribution of a direct G protein effect independent of the cAMP-PKA pathway (or even for any contribution of any other second messenger pathway).

Thus, the inhibitor PKI *completely* abolishes the Iso-activated Cl^- current whether or not GTP (Fig. 4) or GTPγS (Fig. 5) is present, and fully prevents any response to Iso or forskolin (Fig. 4), which means that both responses are *absolutely dependent* upon substrate phosphorylation by PKA. The fact that near maximally effective concentrations of Iso or forskolin activate similar levels of Cl^- conductance (Fig. 7) further supports the notion that both share the same activation pathway. Moreover, during maximal activation of Cl^- conductance by forskolin (Fig. 4) or cAMP,[1] Iso has no effect. A direct, phosphorylation-independent action of G_s on Cl^- channels, via a membrane-delimited pathway, as recently proposed for regulation of a number of

other ion channels (Brown and Birnbaumer, 1988; Brown et al., 1990), is therefore very unlikely.

Furthermore, the fact that the whole-cell *I–V* relationships *c, d,* and *k* in Fig. 5 were identical (see negligible difference *I–V*'s: *B, d − c,* ●, and *D, k − d,* △) means that no Cl^- current, whether PKA dependent or PKA independent, was spontaneously activated during the 15–20 min of intracellular dialysis with 100 μM GTPγS, which should have caused persistent activation of any G protein with a basal turnover rate $\geq \sim 0.1\ min^{-1}$. Thus, although we cannot entirely dismiss the possibility that some G protein other than G_s might affect Cl^- channels directly, all G proteins with a substantial basal turnover are ruled out.

Forskolin Elicits Cl^- Conductance via PKA but without G Protein Activation

The ability of forskolin to elicit the Cl^- conductance in a reversible manner in the absence of GTP, and in the presence of GDPβS (Fig. 7) or GTPγS (Fig. 6), clearly demonstrates that the response to forskolin does not require activation of any G protein. Further, our finding that forskolin has *no effect* after complete inhibition of PKA by a maximally effective concentration of PKI (Fig. 4) means that forskolin activates Cl^- conductance *exclusively* via PKA-mediated channel phosphorylation. This rules out any contribution to the forskolin-induced current from mechanisms that do not involve activation of adenylyl cyclase, like those recently reported to underlie certain effects of forskolin on cation channels (Coombs and Thompson, 1987; Hoshi, Garber, and Aldrich, 1988; Krause, Lee, and Deutsch, 1988; Wagoner and Pallotta, 1988; Zunkler, Trube, and Ohno-Shosaku, 1988). We conclude, therefore, that forskolin acts simply by stimulating adenylyl cyclase.

This conclusion is supported by the observation that forskolin-activated Cl^- current can be diminished by the muscarinic agonists ACh and CCh (Table I, Fig. 9), because it is known that forskolin-induced cAMP accumulation can be inhibited via activation of the G protein, G_i (reviewed by Seamon and Daly, 1986). An analogous inhibition by ACh of forskolin-activated Cl^- current in guinea pig ventricular myocytes was described recently (Tareen, Ono, Noma, and Ehara, 1991; cf. Harvey et al., 1990), and muscarinic agonists have previously been shown to reduce forskolin-activated Ca^{2+} current in guinea pig (Hescheler et al., 1986) and frog (Hartzell and Fischmeister, 1987; Nakajima, Wu, Irisawa, and Giles, 1990; Parsons et al., 1991) myocytes, effects all attributed to forskolin–G_i interaction at the adenylyl cyclase.

Mechanism of Muscarinic Inhibition of Cl^- Current

In contrast to their striking effects on atrial cells, muscarinic agonists elicit virtually no response in ventricular myocytes unless cellular cAMP levels are first elevated. In that case, muscarinic stimulation counters the effects of raised [cAMP], as has been well documented (reviewed by Hartzell, 1988) in the regulation of guinea pig (Hescheler et al., 1986) and frog (Hartzell and Fischmeister, 1987; Nakajima et al., 1990; Parsons et al., 1991) cardiac Ca^{2+} currents. A similar muscarinic inhibition of β-adrenoceptor-mediated Cl^- current has also been reported (Harvey and Hume, 1989; Harvey et al., 1990; Tareen et al., 1991). Possible mechanisms for these muscarinic inhibitory effects include (*a*) activation of a protein phosphatase (e.g., Ahmad, Green, Subuhi, and Watanabe, 1989), (*b*) activation of cGMP-dependent

phosphodiesterase through a rise in cellular [cGMP] (Fischmeister and Hartzell, 1987), or (*c*) inhibition of adenylyl cyclase (e.g., Gilman, 1987).

(*a*) Our results show that near maximally effective concentrations, i.e., 1 μM Iso (bath), 1 μM forskolin (bath), or 1 mM cAMP (pipette), all activate approximately the same level of Cl^- conductance (Figs. 3 and 7), and all do so with an abrupt, abbreviated time course (Figs. 1–3 and 7; Bahinski et al., 1989*a*) that implies saturation of a rate-determining step late in the common pathway. This level of Cl^- conductance evidently reflects a steady-state level of phosphoprotein resulting from continuous activity of an endogenous phosphatase in the face of a maximum rate of PKA-mediated phosphorylation. The strong phosphatase activity is apparent from the rapid decay of Cl^- conductance after withdrawal of Iso (Fig. 2), or application of propranolol (Fig. 1) or PKI (Figs. 4 and 5; Bahinski et al., 1989*a*). That the dephosphorylation rate controls the steady level of Cl^- conductance is clear from the observation that the conductance can be increased, and its decay slowed, by maneuvers expected to interfere with dephosphorylation, such as using ATPγS to phosphorylate or applying the phosphatase inhibitor okadaic acid (Horie, Hwang, and Gadsby, 1991).[1] So, if muscarinic agonists activated a protein phosphatase, then a reduced level of Cl^- conductance would indeed be expected, but the effect should be the same whether Iso, forskolin, or cAMP is the activator. In agreement with Tareen et al. (1991), we find that muscarinic agonists inhibit Cl^- current activated by Iso (Fig. 3) or forskolin (Fig. 9), but not cAMP (Fig. 3), and thus conclude that the muscarinic effect occurs prior to, or at the site of, cAMP generation (cf. Fischmeister and Hartzell, 1986; Hescheler et al., 1986).

(*b*) Although muscarinic agonists can raise cellular [cGMP] in the heart, activation of cGMP-dependent phosphodiesterase is an unlikely mechanism for the muscarinic *inhibition* of Cl^- current, since preliminary results show that pipette perfusion with 100 μM cGMP has practically no effect on Cl^- current elicited by 1 μM Iso (not illustrated). On the contrary, pipette perfusion with cGMP has recently been shown to *enhance* Cl^- current elicited by a submaximal concentration of β-adrenoceptor agonist (Tareen et al., 1991), possibly via *inhibition* of a phosphodiesterase (Ono, Tareen, Yoshida, Noma, and Trautwein, 1991).

(*c*) The most likely mechanism for the muscarinic attenuation of Cl^- current is therefore a direct inhibitory effect on adenylyl cyclase, and this is consistent with all of our results. Our finding that the inhibitory effect was abolished by introduction of GDPβS (Fig. 9, *A* and *B*) or by incubation with PTX (Fig. 9, *C* and *D*) shows that it is mediated by a PTX-sensitive inhibitory G protein, presumably G_i or some other (e.g., G_o) closely related G protein (Gilman, 1987). The question then arises as to whether α subunits of G_i exert a direct inhibitory influence on adenylyl cyclase, or whether released βγ subunits promote, by mass action, the formation of the inactive $\alpha_s\beta\gamma$ complex upon hydrolysis of the GTP bound to the α_s subunit. In cardiac myocytes from the frog, *inhibition* of Iso-stimulated Ca^{2+} currents usually occurs when hydrolysis-resistant GTP analogues are introduced, conditions that should persistently activate α_s, leaving it unavailable for complexation with βγ subunits (Nakajima et al., 1990; Parsons et al., 1991; but cf. Breitwieser and Szabo, 1985); the implication is that the α_i subunits themselves inhibit the adenylyl cyclase. In guinea pig myocytes, on the other hand, we find that GTPγS causes *persistent activation* of Iso-induced Cl^-

current, which is then *resistant* to inhibition by ACh (Fig. 5), presumably because the GTPγS persistently activates G_i and thus uncouples it from the muscarinic receptors. However, Fig. 9, *A* and *B*, demonstrates that the forskolin-induced Cl^- conductance, which does not itself require G protein activation, is also attenuated by muscarinic receptor stimulation, and in a G protein–dependent manner. At least in this case, then, the inhibition of adenylyl cyclase cannot be due to complexation of α_s subunits and probably reflects a direct inhibitory influence of α_i subunits.

Temperature Dependence of PTX Action

The temperature during the incubation with PTX seemed critical (cf. Hescheler et al., 1986), since when we incubated myocytes with 5 μg/ml PTX at room temperature, even for as long as 8 h, inhibition of the Iso-activated Cl^- current by 5 μM ACh was only partially impaired, averaging 25 ± 3% (n = 3) instead of the ~60% seen in control cells. Nakajima et al. (1990) needed to incubate frog atrial myocytes in 0.5 μg/ml PTX for 6 h at 30°C to prevent both ACh-induced inhibition of Iso-activated Ca^{2+} current and ACh-induced activation of K^+ current. They therefore suggested that a temperature dependence might have accounted for the failure, in their earlier report (Shibata, Northup, Momose, and Giles, 1986), of PTX treatment to block muscarinic activation of frog atrial K^+ conductance; possibly, that exposure to PTX was "too short at room temperature." Interestingly, Parsons et al. (1991), also working with frog myocytes, recently reported that even 12–24 h treatment with 0.05–10 μg/ml PTX at room temperature (Hartzell, H. C., personal communication) failed to prevent inhibition of stimulated Ca^{2+} current by ACh. If muscarinic inhibition in frog myocytes is indeed mediated via a PTX-sensitive G protein (Nakajima et al., 1990), as in avian and mammalian myocytes (Fig. 9 above; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Hescheler et al., 1986), then the temperature dependence observed here (and by Hescheler et al., 1986) could account for the negative findings of Parsons et al. (1991) and Shibata et al. (1986). It seems likely that this temperature dependence of PTX action is that of internalization of the holo-toxin, the rate-limiting step (Ui, 1990), because, in experiments on inside-out membrane patches from guinea pig ventricular myocytes, preactivated toxin can exert its full effect within minutes, both at 33–35°C (Ito, Sugimoto, Kobayashi, Takahashi, Katada, Ui, and Kurachi, 1991) and at room temperature (Horie, M., and H. Irisawa, unpublished observations).

Cardiac Cl^- Conductance Is Ideally Suited for In Situ Studies of the Regulation of Adenylyl Cyclase

Although much biochemical work has been done on the regulation of adenylyl cyclase in cell-free reconstituted systems (reviewed in Gilman, 1987), as noted by Parsons et al. (1991) crucial differences between reconstituted systems and intact cells in terms of stoichiometries of the protein components, their relative locations, etc., emphasize the need for functional studies in living cells. Use of a membrane conductance modulated by PKA to monitor adenylyl cyclase activity is a straightforward way to investigate interactions between membrane receptors, G proteins, and adenylyl cyclase. Dihydropyridine-sensitive Ca^{2+} channels have been widely used for this purpose (Breitwieser and Szabo, 1985; Hescheler et al., 1986; Fischmeister and

Shrier, 1989; Nakajima et al., 1990; Shuba et al., 1990; Parsons et al., 1991). Some apparent discrepancies in results from those studies (discussed in Parsons et al., 1991) are possibly attributable to immutable differences between the signaling pathways of frog and guinea pig myocytes, or to different temperature sensitivities of key reactions in those pathways. Others might be attributable to differences in the effective intracellular concentrations of GTP analogues due to varying degrees of diffusional equilibration between pipette solutions and the cell interior.

In several respects, the PKA-regulated Cl^- current seems more suitable than Ca^{2+} current as a monitor of adenylyl cyclase activity in a mammalian cell. Thus, the Cl^- conductance, like the open probability of the underlying Cl^- channels (Ehara and Ishihara, 1990), is time and voltage independent (Bahinski et al., 1989*b*), which means that Cl^- current at a fixed membrane potential can monitor faithfully the time course of cellular events. Whereas Ca^{2+} channels are modulated by phosphorylation but gated by voltage, phosphorylation appears to be obligatory for gating of the Cl^- conductance, since it is abolished by PKI and is absent under basal conditions (Fig. 4, above; Bahinski et al., 1989*b*). As long as pipette solutions contain GTP (Horie et al., 1992), the Cl^- conductance is less subject to rundown than Ca^{2+} currents (e.g., Belles et al., 1988) so that, as demonstrated here, wide-tipped pipettes (even with resistances <1 MΩ) can be used to provide maximum control over the intracellular environment.

Using this approach, we have begun to examine the interactions between G_s, G_i, and forskolin in the regulation of mammalian adenylyl cyclase. Although forskolin has been shown to activate adenylyl cyclase in the absence of functional G_s (e.g., Seamon and Daly, 1986), and our results here confirm that G protein turnover is not required for forskolin action, its effect on adenylyl cyclase does appear to be strongly influenced by the activities of both G_s and G_i. For example, the action of forskolin can be potentiated by low concentrations of agonists whose receptors are coupled to G_s, whereas it is attenuated by hormones that stimulate G_i (Seamon and Daly, 1986). Parsons et al. (1991), using frog cardiac Ca^{2+} current to monitor adenylyl cyclase activity, have recently reported that Iso in the presence of GppNHp increased the apparent affinity for forskolin, while ACh, or GppNHp alone, lowered it. Our preliminary findings (Hwang, Horie, Dousmanis, and Gadsby, 1992), using Cl^- conductance in guinea pig myocytes, of a reduction of apparent forskolin affinity by pipette GTPγS alone, of occlusion by GTPγS of the similar effect caused by CCh, and of enhancement of apparent forskolin affinity by Iso, corroborate the findings in frog myocytes. The implications are that activated G_s and G_i alter the affinity of adenylyl cyclase for forskolin, and that nonhydrolyzable GTP analogues in the absence of receptor agonists preferentially activate G_i. Our observation that GTPγS alone does not activate Cl^- conductance is also consistent with this latter suggestion. Possible reasons for its preferential activation are that the affinity of G_i for GTP analogues is higher than that of G_s (Jakobs, Aktories, Minuth, and Schultz, 1985), that the basal turnover rate of G_i is greater than that of G_s, and/or that the overall density of G_i in the myocyte membrane is greater than that of G_s (see discussion in Parsons et al., 1991).

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