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ADENYLYL CYCLASE INTEGRATES MULTIPLE G PROTEIN SIGNALS TO MODULATE CALCIUM CURRENTS IN NEONATAL RABBIT HEART*

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SUMMARY: We investigated the effects of added $\beta\gamma$ subunits of G proteins ($G\beta\gamma$) on β-adrenergic responsiveness of transmembrane Ca^{2+} currents (I_{Ca}) in ventricular myocytes from neonatal rabbits. $G\beta_1\gamma_1$ purified from retinal rods was dialyzed into cells via the voltage clamp micro-electrode. Stimulation of I_{Ca} by isoproterenol was not affected by added intracellular $G\beta_1\gamma_1$ or by carbachol alone but was completely blocked by combined $G\beta_1\gamma_1$ and carbachol. Pretreatment of cells with pertussis toxin or temporal separation of carbachol and isoproterenol allowed stimulation of I_{Ca} by isoproterenol in cells dialyzed with $G\beta_1\gamma_1$. Carbachol and $G\beta_1\gamma_1$ together also did not prevent stimulation of I_{Ca} by dibutyryl-cyclic AMP. Thus, rather than simply inactivating $G_s\alpha$ by mass action, $G\beta_1\gamma_1$ acts in concert with carbachol to inhibit isoproterenol stimulation of I_{Ca}.

Cardiac contractility in mammals is modulated by beat-to-beat changes in intracellular calcium ion concentration. Newborn mammal hearts depend on transmembrane Ca^{2+} influx to provide Ca^{2+} ions for contraction [1], since they lack sarcoplasmic reticulum (SR) [1] . β-Adrenergic enhanced contractility in mature heart muscle is largely mediated by activation of protein kinase A (PKA), which phosphorylates phospholamban [2], troponin I [2], and the L-type calcium channel [3]. Neonatal heart cells are well-suited for investigating the direct modulatory effects of β-adrenergic stimulation on Ca^{2+} current (I_{Ca}) due to the under-

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development of SR, phospholamban and troponin I [4]. The α subunits of G protein (Gα), which contain the guanine nucleotide-binding site, are specific to each G protein, while the $\beta\gamma$-subunit dimers (G$\beta\gamma$) appear to be shared to some extent among G proteins. Recent studies, however, indicate that G$\beta\gamma$ subunits directly activate a number of intracellular effector proteins [5-10]. We therefore altered intracellular concentration of a specific G$\beta\gamma$ to assess its effects on isoproterenol-stimulated I_{Ca} in freshly isolated ventricular myocytes from neonatal rabbits.

METHODS AND MATERIALS

Cell isolation and measurement of I_{Ca}. Ventricular myocytes were isolated from neonatal (2-5 day old) New Zealand white rabbits (50-125 g) by enzymatic dissociation [11] and in accordance with institutional guidelines. Ventricular cells were then placed in a recording chamber ($\sim$1.5 mL) on the stage of a Diaphot inverted microscope (Nikon, Japan). The whole cell voltage clamp technique previously described [12] was used. I_{Ca} was recorded using Corning 8161 (Corning Glass Co., Horsehead, NY) glass micro-electrodes filled with internal solution containing 110 mM CsOH, 10 mM tetraethylammonium-Cl, 10 mM EGTA with 5 mM $CaCl_2$ (to buffer $[Ca^{2+}]$ to $\sim$0.3 μM), 10 mM CsCl, 90 mM aspartic acid, 5 mM Cs-Hepes pH 7.1, 5 mM Na_2-creatine phosphate, 0.4 mM Tris-GTP, 0.1 mM leupeptin, and 5 mM Mg-ATP. External bath solution contained 130 mM NaCl, 20 mM CsCl, 5 mM Na-Hepes pH 7.3, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5 mM glucose, and 0.01 mM tetrodotoxin. All experiments were performed at 24°C. In some experiments, isolated ventricular myocytes were incubated for 2 hr at 30°C in Tyrode's solution containing 0.1 mM $CaCl_2$ with 5 μg/ml pertussis toxin (List Biological, Campbell, CA).

Purification of $\beta\gamma$-subunit of retinal rod transducin. The $\beta\gamma$-subunits of retinal rod transducin ($G\beta_1\gamma_1$) were purified from frozen bovine retinas as part of the phosducin/G$\beta\gamma$ complex and subsequently dissociated from phosducin as described previously [13]. $G\beta_3\gamma$ was removed during purification by sequential chromatography [13,14]. Purified $G\beta_1\gamma_1$ was concentrated by ultrafiltration using an Amicon YM-10 membrane and stored at -20°C in a buffer containing 10 mM Na-3-[N-morpholino]propanesulfonate (pH 7.5), 0.2 M NaCl, 0.2 mM EDTA 2 mM dithiothreitol, and 40% (w/v) glycerol (storage solution). The final concentration of $G\beta_1\gamma_1$ in the storage solution was $\sim$0.5 mM. All control experiments were performed using micro-electrode solution identical to the experimental solution but without $G\beta_1\gamma_1$. $G\beta_1\gamma_1$ was added to ventricular myocytes by diffusion from the micro-electrode. For proteins with molecular weight $\sim$40,000 (*i.e.*, $G\beta_1\gamma_1$), the calculated time constant for diffusion into a neonatal cardiac cell is $\sim$5 min [15]. Therefore, all experiments were performed $>$10 min after seal formation.

Statistical analyses. Data for groups are presented in the text as mean $\pm$ standard error of the mean (number of cells). Statistical significance was determined by two-tailed Student t-test. P values <0.05 were considered significant.

RESULTS

Effects of $G\beta_1\gamma_1$ on isoproterenol-stimulated I_{Ca}. In neonatal ventricular myocytes with added intracellular $G\beta_1\gamma_1$, isoproterenol increased I_{Ca} both 10 min and 30 min after seal formation (Fig. 1A). At 10 min, the maximal peak I_{Ca} was 3.1$\pm$0.5 pA/pF and increased to 6.3$\pm$0.2 pA/pF (n=4) with addition of 10 μM isoproterenol. Similarly, at 30 min, maximal

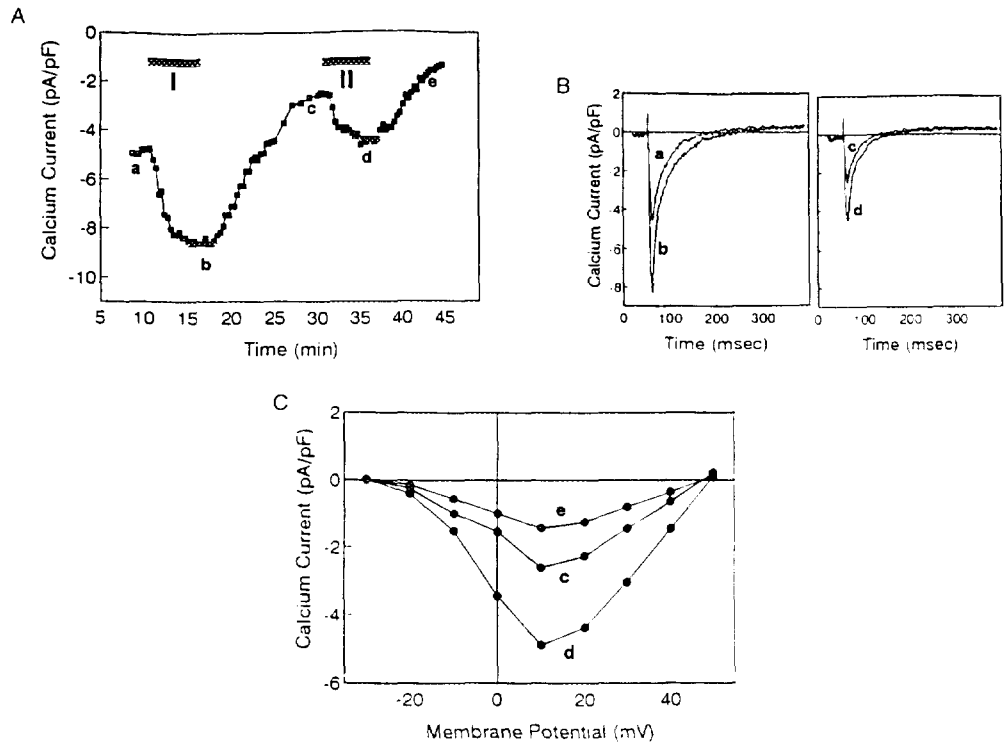

Figure 1. Effects of isoproterenol and added intracellular $G\beta_1\gamma_1$ on I_{Ca} in a typical neonatal rabbit ventricular myocyte. Seal formation between the electrode containing 1 μM $G\beta_1\gamma_1$ and the cell interior occurred at time = 0. Voltage clamp steps were applied every 20 sec from a holding potential of -80 mV to a prepulse potential of -40 mV for 50 msec and then to a test potential of +10 mV for 400 msec. At 10 min and 30 min after seal formation, the myocyte was exposed to 10 μM isoproterenol for 5 min (shown as solid bars labeled I and II in panel A). (A) Graph of peak I_{Ca} *vs.* time. The steady decline in I_{Ca} seen during this experiment is typical "rundown" of I_{Ca} which occurs in neonatal heart cells. (B) Original traces of I_{Ca} at times *a*, *b*, *c*, and *d* indicated in panel *A*. (C) Current-voltage relations at time points *c*, *d*, and *e* indicated in *A*.

peak I_{Ca} was 1.3±0.2 pA/pF and increased to 2.5±0.5 pA/pF (n=4) with addition of 10 μM isoproterenol. The time and voltage dependence of I_{Ca} was unaffected by diffusion of $G\beta_1\gamma_1$ into the cell between 10 and 30 min (Figs. 1B and 1C). Control experiments without $G\beta_1\gamma_1$ in the micro-electrode were performed and the dependence of I_{Ca} on time and voltage was similar to that previously reported [12,16]. Thus added intracellular $G\beta_1\gamma_1$ does not directly attenuate the stimulatory effects of β-adrenergic-activated α-subunit of G_s ($G_s\alpha$) on I_{Ca}.

Effects of $G\beta_1\gamma_1$ in the presence of carbachol. The muscarinic agonist carbachol antagonizes the effects of isoproterenol by activating inhibitory G proteins (G_i). In order to investigate possible interactions between $G\beta_1\gamma_1$, $G_s\alpha$, and the α-subunits of G_i ($G_i\alpha$), 10 μM carbachol was added prior to and during stimulation of I_{Ca} by isoproterenol. Without added intracellular $G\beta_1\gamma_1$, 10 μM isoproterenol in the presence of carbachol increased I_{Ca}

(isoproterenol-induced increase in I_{Ca}: 1.9± 0.8 pA/pF (n=6); see Figs. 2A and 2B). In contrast, the combination of carbachol and 40 nM intracellular $G\beta_1\gamma_1$ completely blocked the isoproterenol-stimulated increase in I_{Ca} (isoproterenol-induced increase in I_{Ca} with added $G\beta_1\gamma_1$: -0.1±0.1 pA/pF (n=6); p=0.02 *vs.* without added $G\beta_1\gamma_1$; see Figs. 2C and 2D). This finding was unexpected since neither alone appears to alter the response of I_{Ca} to isoproterenol. These results may be explained by hypothesizing that both $G_i\alpha$ activation and *$G\beta_1\gamma_1$ must be present in order to block the effect of isoproterenol on I_{Ca}.*

Temporal separation of carbachol and isoproterenol treatments. Further experiments were performed to test whether carbachol exposure facilitated exchange of $G_i\beta\gamma$ bound to $G_i\alpha$ for the added $G\beta_1\gamma_1$ to liberate $G_i\beta\gamma$ which might then block the response to isoproterenol by binding activated $G_s\alpha$, as originally proposed for explaining the inhibitory effects of $G\beta\gamma$ on adenylyl cyclase [17]. We found that with added intracellular $G\beta_1\gamma_1$,

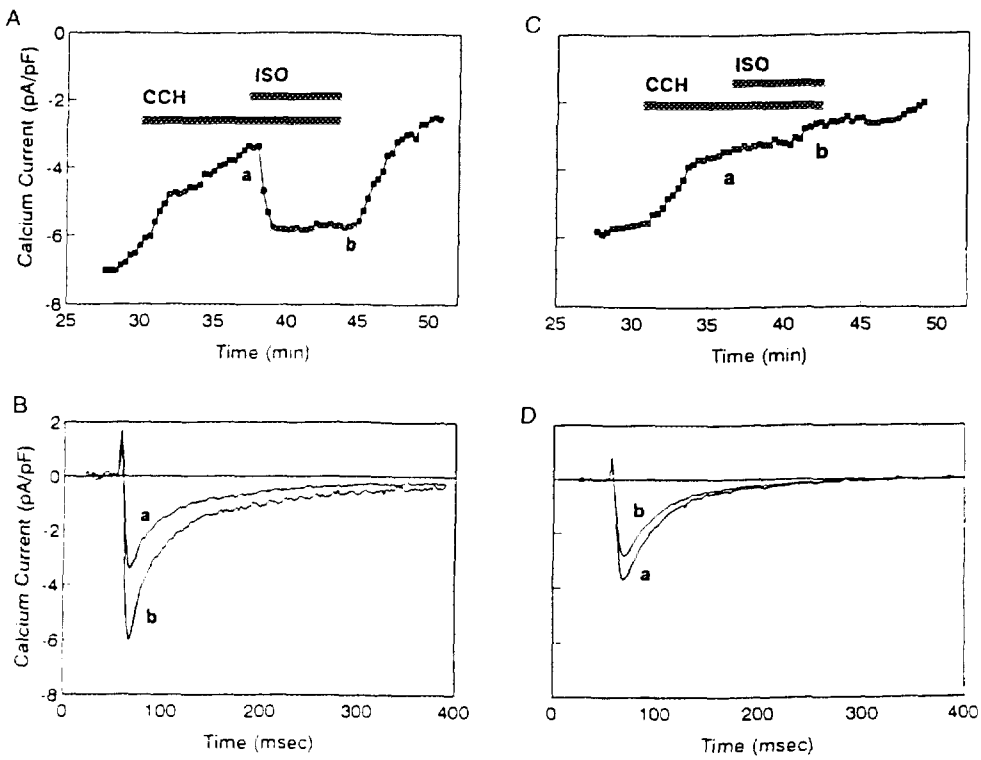

Figure 2. Effect of added intracellular $G\beta_1\gamma_1$ on isoproterenol stimulation of I_{Ca} in typical cardiomyocytes during treatment with carbachol. The upper panels show a plot of peak I_{Ca} *vs.* time in representative cardiomyocytes dialyzed with micro-electrode solution lacking (**A**) or containing 40 nM $G\beta_1\gamma_1$ (**C**). Measurements were started 25 min after seal formation between the electrode and cell interior was established (abscissa indicates time after seal formation). Addition of 10 μM carbachol (**CCH**) and 10 μM isoproterenol (**ISO**) to the extracellular perfusion solution is indicated by bold horizontal lines. Panels **B** and **D** display original records of I_{Ca} at points *a* and *b* indicated in panels **A** and **C**, respectively.

discontinuation of carbachol perfusion 5 min prior to addition of isoproterenol had no effect on stimulation of I_{Ca} by isoproterenol (Fig. 3). This experiment indicates that the cell has no "memory" for the prior exposure to carbachol, and suggests that $G\beta_1\gamma_1$ does not form a stable heterotrimer with $G_i\alpha$-GDP.

Effects of pertussis toxin. Pertussis toxin ADP-ribosylates $G_i\alpha$ in its heterotrimeric state thereby preventing its activation by ligand-muscarinic receptor complex. We found that when $G\beta_1\gamma_1$ was introduced into cells that had been pre-treated with pertussis toxin, carbachol no longer blocked the stimulatory effect of isoproterenol on I_{Ca} (Fig. 4). These data support the notion that $G\beta_1\gamma_1$ and activated $G_i\alpha$ act synergistically to block stimulation of I_{Ca} by isoproterenol, while neither alone can cause this effect. In addition, neither G proteins which are not substrates for pertussis toxin nor the activated muscarinic receptor appear to affect this inhibitory process.

Effects of dibutyryl-cyclic AMP. Our results suggest an integration of signals modulated by $G_s\alpha$ and $G_i\alpha$ that is mediated by excess intracellular $G\beta_1\gamma_1$. In order to determine the level of the cellular signaling cascade at which integration of these G protein signals occurs, we employed N^6,2'-O-dibutyryladenosine 3':5'-monophosphate (dibutyryl-cyclic AMP), a cyclic AMP analog that crosses the cell membrane and directly activates PKA. We found that dibutyryl-cyclic AMP stimulated I_{Ca} and this effect was not blocked by carbachol and added intracellular $G\beta_1\gamma_1$ (Fig. 5). Similar results were obtained in the Ca^{2+} current measurement using three different concentrations of $G\beta_1\gamma_1$ ------ 40 nM, 100 nM and 1 μM. These results suggest that integration of signals modulated by $G_s\alpha$ and $G_i\alpha$ affected by $G\beta_1\gamma_1$ occurs prior to the synthesis of cyclic AMP in neonatal cardiomyocytes.

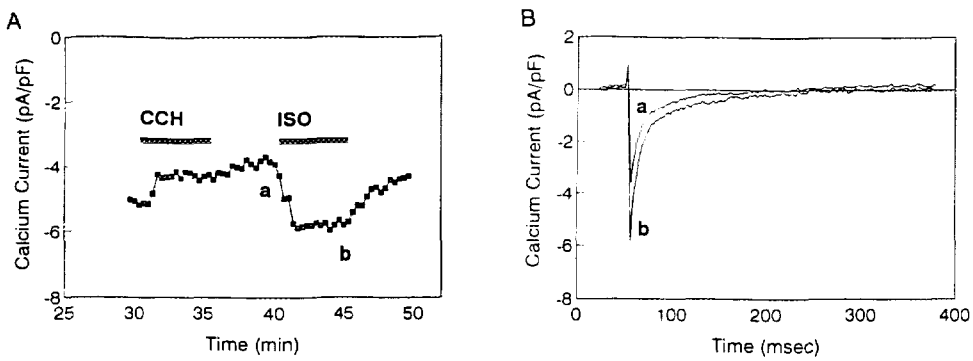

Figure 3. Peak I_{Ca} in typical cardiomyocytes with added intracellular $G\beta_1\gamma_1$ separately treated with carbachol and isoproterenol. **(A)** The effect of 10 μM carbachol **(CCH)** added 30 min after seal formation between the cell and a micro-electrode containing electrode solution with added 1 μM $G\beta_1\gamma_1$. Five min after washout of carbachol, 10 μM isoproterenol **(ISO)** was added. **(B)** Original records of I_{Ca} at time points *a* and *b* indicated in panel **A**.

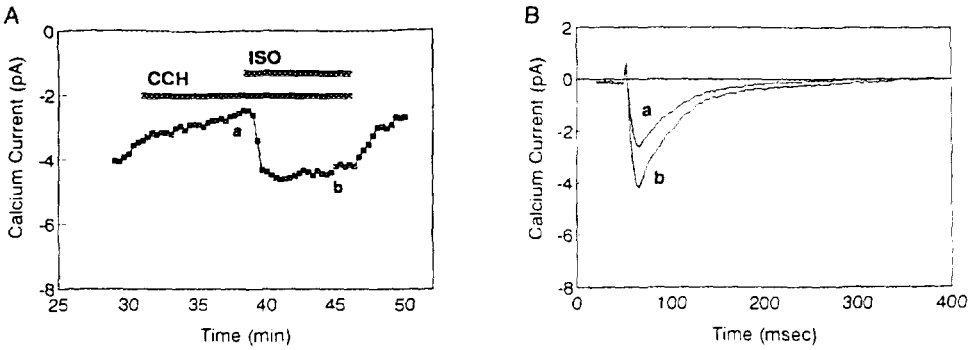

Figure 4. Peak I_{Ca} in typical cardiomyocytes pre-treated with pertussis toxin. **(A)** Plot of peak I_{Ca} *vs.* time after seal formation in cardiomyocytes pre-treated with pertussis toxin prior to the experiment with 1 μM $G\beta_1\gamma_1$ in the micro-electrode. **(B)** Original traces of I_{Ca} at times *a* and *b* indicated in panel **A**.

DISCUSSION

We investigated the effects of added intracellular $G\beta_1\gamma_1$ on β-adrenergic signal transduction in ventricular myocytes freshly isolated from neonatal rabbits. The combination of added $G\beta_1\gamma_1$ and the muscarinic agonist, carbachol, completely blocked β-adrenergic stimulation of I_{Ca}. By contrast, stimulation of I_{Ca} by dibutyryl-cyclic AMP was unaffected by the combination of carbachol and added intracellular $G\beta_1\gamma_1$.

In an adenylyl cyclase system reconstituted from bovine caudate nucleus [18], retinal $G\beta\gamma$ was as effective as brain $G\beta\gamma$ for inhibiting adenylyl cyclase, although ~100x higher concentrations of retinal $G\beta\gamma$ were required. In addition, Muller *et al.* [10] demonstrated that retinal $G\beta\gamma$ can combine with $G_o\alpha$ from bovine brain to facilitate ADP-ribosylation of $G_o\alpha$ by pertussis toxin. Therefore, we used $G\beta_1\gamma_1$ isolated from retinal rods as a probe to study

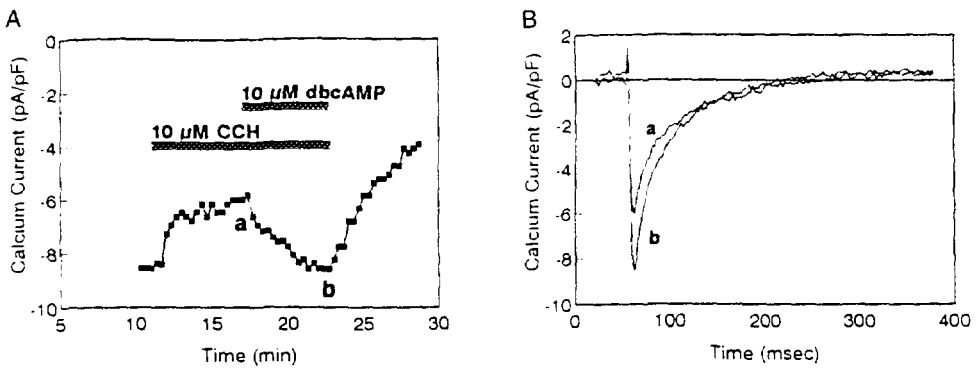

Figure 5. Effects of dibutyryl-cyclic AMP (dbcAMP) on peak I_{Ca} in a typical cardiomyocyte with 100 nM added intracellular $G\beta_1\gamma_1$. (A) Plot of maximal peak I_{Ca} *vs.* time after seal formation. **(B)** Original traces of I_{Ca} at times *a* and *b* indicated in panel **A**.

the functional role of $G\beta_1\gamma_1$ in modulating β-adrenergic responsiveness in the heart. However, added intracellular $G\beta_1\gamma_1$ alone did not inhibit the isoproterenol response of I_{Ca} to isoproterenol to any great extent (Fig. 1A), even at high concentrations similar to those found to be effective by Cerione *et al.* [18] for inhibiting G_s-activated adenylyl cyclase from caudate nucleus. The undetectable effect of $G\beta_1\gamma_1$ on I_{Ca} in cardiac cells stimulated by isoproterenol makes unlikely the possibility that $G\beta_1\gamma_1$ promotes inactivation of activated $G_s\alpha$ by mass action. In reconstituted phospholipid membranes, retinal $G\beta\gamma$ has been shown to down-regulate β-adrenergic receptors by activating β-adrenergic receptor kinase [10]. However, activation of β-adrenergic receptor kinase by added $G\beta_1\gamma_1$ seems unlikely since added intracellular $G\beta_1\gamma_1$ alone had minimal effects on β-adrenergic stimulation of I_{Ca}. In addition, the enzyme β-adrenergic receptor kinase is only weakly stimulated by retinal $G\beta\gamma$ in a reconstituted system [10,19]. These results suggest that $G\beta_1\gamma_1$ does not inhibit effects of activated $G_s\alpha$ directly or activate β-adrenergic receptor kinase.

The present study showed the cooperative inhibition of isoproterenol-stimulated I_{Ca} by added intracellular $G\beta_1\gamma_1$ and the muscarinic agonist, carbachol (Fig. 2). The abolition of this cooperative inhibition, either by temporal separation of carbachol and isoproterenol exposure (Fig. 3) or by pretreatment with pertussis toxin (Fig. 4), suggests that both $G\beta_1\gamma_1$ and muscarinic stimulation are necessary to inhibit isoproterenol stimulation of I_{Ca}. This interaction between muscarinic stimulation and added intracellular $G\beta_1\gamma_1$ is similar to the coincidence detector model [20] to regulate isoform II of adenylyl cyclase in neural cells and the N-methyl-D-aspartate receptor in neuronal networks. In the model, cells "detect two or more temporally coincident input signals and generate an output signal that differs from the output generated in response to a single input signal." In the case of neonatal cardiomyocytes, activation of muscarinic receptor or addition of intracellular $G\beta\gamma$ alone minimally affect isoproterenol-stimulated I_{Ca} (cell output), but the coincidence of these two signals produces a large effect.

We added dibutyryl-cyclic AMP, a cyclic AMP analog that crosses the cell membrane and directly activates PKA. Unlike isoproterenol, dibutyryl-cyclic AMP stimulated I_{Ca} in the presence of carbachol and added intracellular $G\beta_1\gamma_1$ (Fig. 5). These results indicate that signal integration occurs prior to cyclic AMP synthesis. Possible mechanisms include: 1) cardiac adenylyl cyclase integrates signals mediated by activated $G_s\alpha$, activated $G_i\alpha$, and $G\beta\gamma$; 2) $G\beta_1\gamma_1$ associates transiently with de-activated $G_i\alpha$ (after hydrolysis of GTP to GDP), leaving $G_i\beta\gamma$ to block the action of $G_s\alpha$ by mass action as originally proposed by Gilman [17,21]; and 3) $G\beta_1\gamma_1$ alters desensitization of the muscarinic receptor, enhancing muscarinic inhibition of cyclic AMP synthesis. Our results suggest that altered intracellular concentrations of $G\beta\gamma$ isoforms play a major role in integrating β-adrenergic and muscarinic

effects on I_{Ca}. In addition, integration of G protein-mediated signals occurs prior to synthesis of cyclic AMP. These interactions may provide important mechanisms in pathologic decreased cardiac function.

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