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ACCELERATED COMMUNICATION

Calcium Entry via L-Type Calcium Channels Acts as a Negative Regulator of Adenylyl Cyclase Activity and Cyclic AMP Levels in Cardiac Myocytes

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SUMMARY

It is well established that the inotropic effect of β-adrenergic agonists is mediated by the stimulation of adenylyl cyclase activity and the subsequent phosphorylation of specific proteins by cAMP-dependent protein kinase. The L-type calcium channel is believed to be one of the proteins phosphorylated; the phosphorylation of calcium channels is believed to increase calcium entry into myocytes, which is, at least in part, responsible for the positive inotropic effect. The present studies show that the cAMP-elevating effect of isoproterenol is increased as extracellular calcium is lowered and that calcium channel blockers potentiate the cAMP-elevating effect of isoproterenol in the presence in extracellular calcium. This effect is not dependent on effects on cAMP catabolism and is not specific for β-adrenergic receptors, because the cAMP-elevating effect of forskolin is similarly affected. Measurements of adenylyl cyclase activity in cardiac membranes show that submicromolar Ca^{2+} concentrations directly inhibit adenylyl cyclase activity. These results demonstrate that increased entry of Ca^{2+} via L-type calcium channels in response to β-adrenergic receptor stimulation acts as a negative regulator of the effect of β receptor stimulation on adenylyl cyclase activity.

The abilities of numerous receptor types to couple to the inhibition and stimulation of adenylyl cyclase activity in a single cell type was the first indication of the complex nature of the control of cAMP production. The discovery that multiple types of G proteins "couple" receptors to adenylyl cyclase further increased the complexity of the system (reviewed in Ref. 1). This complexity was further advanced by the discovery of the existence of multiple types of adenylyl cyclase (reviewed in Ref. 2). Although the existence of Ca^{2+}/calmodulin-sensitive and -insensitive forms of adenylyl cyclase was long suspected, the existence of other types of adenylyl cyclase with importantly different regulatory characteristics was rather unexpected (2).

Although it was clear a number of years ago that Ca^{2+} inhibits rather than stimulates cardiac adenylyl cyclase, the involvement of calmodulin in this effect was unclear (3). The finding that mRNAs for Ca^{2+}/calmodulin-activated (type 1) adenylyl cyclase and the first type of Ca^{2+}/calmodulin-insensitive adenylyl cyclase that was cloned (type II) were not detectable in the heart suggested that cardiac adenylyl cyclase is of yet another type (4). Recently, four laboratories have cloned and expressed adenylyl cyclase isoforms (types V and VI) that are highly expressed in the heart (4–8).

Although types V and VI appear to be the major isoforms of adenylyl cyclase expressed in the heart, they are not cardiac specific, inasmuch as they are expressed in other tissues and cells (4–8). A very interesting characteristic of these adenylyl cyclase isoforms is that they are inhibited by Ca^{2+} in the same concentration range that activates the Ca^{2+}/calmodulin-stimulated adenylyl cyclase (8). [Actually, accurate determinations of Ca^{2+} sensitivity have been reported only for type VI adenylyl cyclase, and similar sensitivity for type V is inferred from studies in which free Ca^{2+} concentrations were not calculated (3–5).]. Importantly, it has now been shown that receptor-mediated elevations in $[Ca^{2+}]_i$ can inhibit cAMP accumulation in intact cells (9–12). As pointed out by both Krupinski *et al.*

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular calcium concentration; ISO, isoproterenol; PDE, phosphodiesterase; IBMX, isobutylmethylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; (*R*)-PIA, (*R*)-N^6-(2-phenylisopropyl)adenosine.

(6) and Cooper and Brooker (13), this raises an interesting possibility in cardiac tissue, a tissue in which cAMP plays an important role in Ca^{2+} homeostasis. It is well known that stimulation of cardiac β-adrenergic receptors stimulates adenylyl cyclase activity and elevates cAMP, and it is believed that this elevation in cAMP modifies L-type calcium channel activity, which in turn causes an elevation in $[Ca^{2+}]_i$ and an increase in the inotropic state of the heart (14). Thus, it is possible that the effect of β agonists on L-type Ca^{2+} channel activity causes an increase in $[Ca^{2+}]_i$ sufficient to inhibit adenylyl cyclase and thus act as a negative regulator of this important pathway. The results reported herein clearly demonstrate that this negative regulatory pathway plays an important role in regulating adenylyl cyclase activity and consequently cAMP levels in embryonic chick cardiac myocytes.

Experimental Procedures

Materials. Fertilized White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Nifedipine and (±)-Bay K-8644 were obtained from Research Biochemcials, Inc. (Natick, MA). D-600, milrinone, and rolipram were gifts from Knoll Pharmaceutical Co. (Whippany, NJ), Sterling-Winthrop Research Institute (Rensselear, NY), and Berlex Laboratories, Inc. (Cedar Knolls, NJ), respectively. Pertussis toxin was from List Biologicals (Campbell, CA). Tissue culture supplies were from Biologos Inc. (Naperville, IL), GIBCO/BRL (Grand Island, NY), and Celox (Hopkins, MN). [^{3}H]cAMP, [^{3}H]adenine, and [^{32}P]ATP were from ICN (Irvine, CA). [^{14}C]cAMP was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation of embryonic chick ventricular myocytes and cell culture. Ventricular cardiac myocytes were isolated from 11–13-day-old chick embryos using multiple rounds of trypsinization, as described previously (15). Cells were cultured in 24-well culture dishes in a M199-based medium as described previously (15), with the exception that the fetal bovine serum was replaced by 0.5% chicken serum, 1% lipid concentrate (catalogue number 680–1900AG; GIBCO), and 2% serum supplement TM-235 (Celox). Cells cultured in this medium were more predictably responsive to agonists that act at G protein-coupled receptors than were cells cultured in fetal bovine serum-containing medium.[1]

Measurement of cAMP accumulation in intact myocytes. Cells maintained in culture for 3–4 days were used for all experiments. Relative changes in cAMP levels were determined by measuring the conversion of [^{3}H]ATP to [^{3}H]cAMP using the [^{3}H]adenine technique (16–17), as used previously in this laboratory (15). Briefly, cells were incubated (37°, 1 hr) in medium containing [^{3}H]adenine (2 μCi/ml), 140 mM NaCl, 5 mM KCl, 1.0 mM $MgSO_4$, 0.45 mM $CaCl_2$, 25 mM HEPES (adjusted to pH 7.4 with Tris base), and 5 mM glucose. The cells were then washed once and placed in the same medium containing adenosine deaminase (2 units/ml). After 6 min of incubation at 37°, an equal volume of drug-containing solution (temperature equilibrated) was added and the incubation was continued for 6 min. All drug solutions contained the PDE inhibitors milrinone (10 μM final concentration) and rolipram (100 μM final concentration). Incubations were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. Samples were processed as described previously, using [^{14}C]cAMP to correct for recovery (15). The results are expressed as percentage conversion of [^{3}H]ATP to [^{3}H]cAMP. Changes in percentage conversion are interpreted as changes in cAMP levels and are sometimes referred to as such. All experiments were performed in quadruplicate. Samples studied in nominally Ca^{2+}-free solution were labeled in the same solution and subsequently studied in the same solution but with no $CaCl_2$ and 200 μM EGTA.

Assay of adenylyl cyclase activity. Crude membrane preparations were prepared from frozen (in liquid nitrogen) dissociated ventricular myocytes. Cells were washed twice in lysis buffer (25 mM HEPES (adjusted to pH 7.4 with NaOH), 2 mM EGTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml soybean trypsin inhibitor), using centrifugation at 350 × g for 4 min to pellet the cells, and were allowed to swell for 15 min on ice before being disrupted by passage through increasingly small syringe needles. The cell "homogenate" was pelleted by centrifugation at 30,000 × g for 20 min and was resuspended in 10 mM HEPES, pH 7.4, with a syringe and needle, to give a protein concentration of 4–5 mg/ml. Adenylyl cyclase activity was assayed at 30° in an incubation reaction containing 4 mM phosphocreatine, 20 units/ml creatine phosphokinase, 1 mM $MgSO_4$, 0.1 mM ATP (disodium salt), [α-^{32}P]ATP (2–3 × 10^6 cpm/tube), 2 mM EGTA, 25 mM HEPES pH 7.4, and 20 μM forskolin. [^{32}P]cAMP was isolated and quantitated using standard techniques (18). Total $CaCl_2$ added was varied to give the theoretical free Ca^{2+} concentrations given. These values, which are adjusted for the effects of the reaction constituents on free Ca^{2+} concentrations, were calculated using a reiterative computer program (19). Assays were performed in triplicate under conditions in which product formation was linear with time and protein concentration.

Miscellaneous methods. Protein was assayed by the Coomassie Blue G-250 dye-binding assay, using a commercial reagent (Pierce, Rockford, IL). Curves were analyzed and graphics were produced using GraphPAD (ISI Software, Philadelphia, PA).

Results and Discussion

We have previously reported studies on cAMP levels in embryonic chick myocytes (15). These studies were performed using an incubation buffer solution containing 0.45 mM $CaCl_2$. This lower than "normal" $CaCl_2$ concentration was used because the cAMP-elevating effect of ISO was more robust in this solution, compared with that obtained in solutions containing normal (1.5–2 mM) $CaCl_2$ concentrations.[2] The discovery that mammalian cardiac tissue contains adenylyl cyclase isozymes that are inhibited by low Ca^{2+} concentrations prompted us to recall this observation and to perform experiments to evaluate its significance. The working hypothesis was that elevations in $[Ca^{2+}]_i$ that occur subsequent to β-adrenergic receptor stimulation, activation of adenylyl cyclase, and acti-

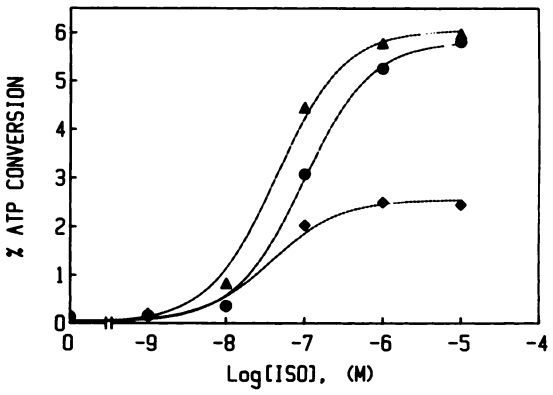

Fig. 1. Dose-response curves for ISO in 0.45 mM $CaCl_2$ in the absence (♦) and presence (●) of D-600 (100 μM) and in 0 mM calcium (▲). The results shown are from one of three experiments, which gave similar results.

[1] H. J. Yu and R. D. Green. Maintenance of receptor function in embryonic chick cardial myocytes in primary culture. Manuscript in preparation.

[2] H. Ma and R. D. Green, unpublished observations.

vation of L-type Ca^{2+} channels are sufficient to act as a negative regulator of adenylyl cyclase activity.

Fig. 1 shows a dose-response curve for ISO in the presence of 0.45 mM $CaCl_2$ (♦) and in nominally Ca^{2+}-free solution (▲). Clearly, the response to ISO in the lower than normal Ca^{2+} solution is blunted, compared with that in Ca^{2+}-free solution. If the effect of the extracellular Ca^{2+} on the response to ISO is mediated by Ca^{2+} entering the cells via L-type Ca channels, then the effect of ISO in the presence of extracellular Ca^{2+} should be increased in the presence of a calcium channel blocker. The experiment shown in Fig. 1 contains a group in which the calcium channel blocker D-600 (100 μM) was present (●). The dose-response curve for ISO in the presence of 0.45 mM $CaCl_2$ plus D-600 was similar to that in the absence of $CaCl_2$, suggesting that the effect of D-600 was due to the blockade of calcium channels. The experiment in Fig. 2 shows that the dihydropyridine calcium channel blocker nifedipine had the same potentiating effect and that neither agent had an effect in the absence of extracellular calcium. Thus, the effects of the calcium channel blockers to increase the cAMP-elevating effect of ISO appear to be due to the blockade of Ca^{2+} entry via L-type calcium channels. It should also be noted that the rather high concentration of D-600 used in the experiments shown (100 μM) was not necessary, in that 1 μM D-600 had the same potentiating effect (data not shown).

If calcium channel blockers potentiate the effect of ISO on cAMP levels by blocking the entry of Ca^{2+} through L-type calcium channels, then one would predict that the dihydropyridine calcium channel agonist Bay K-8644 would depress the dose-response curve for ISO. The experiment shown in Fig. 3 shows that this was indeed the case.

There are several possible mechanisms that could underlie the effects of extracellular Ca^{2+} and the calcium channel antagonists on the elevation of cAMP in response to ISO. One possibility is an effect on G_i function. This possibility was ruled out by showing that the effect of Bay K-8644 was unaffected by pretreatment of the cells overnight with pertussis toxin (Fig. 4). A group treated with the A_1 adenosine receptor agonist (*R*)-PIA was included as a positive control, i.e., to verify that a response mediated by a pertussis toxin-sensitive G protein was blocked. A second possibility is that the effect is specific for β receptor stimulation. This possibility is inconsistent with the working hypothesis, which would predict that any treatment that elevates cAMP levels would activate calcium channel activity and thus feed back on the cAMP elevation. The experiment summarized in Fig. 5 shows that increasing $CaCl_2$ concentrations lowered both ISO- and forskolin-elevated cAMP levels in a concentration-dependent manner. Thus, the effect is clearly independent of any effect on β receptors *per se*. (The possible significance of the apparently greater depression of ISO-elevated cAMP, compared with forskolin-elevated cAMP, by high concentrations of $CaCl_2$ is unknown at present.) Furthermore, additional samples in the same experiment (Fig. 5, *bar graphs*) show that the effects of nifedipine and Bay K-8644

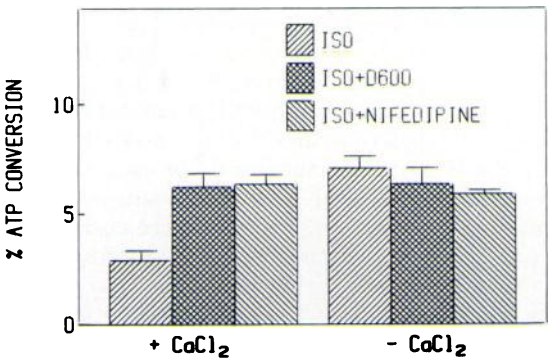

Fig. 2. Effects of D-600 (100 μM) and nifedipine (1 μM) on ISO (10 μM)-elevated cAMP levels in 0.45 mM and 0 mM $CaCl_2$. Results are from one of two experiments.

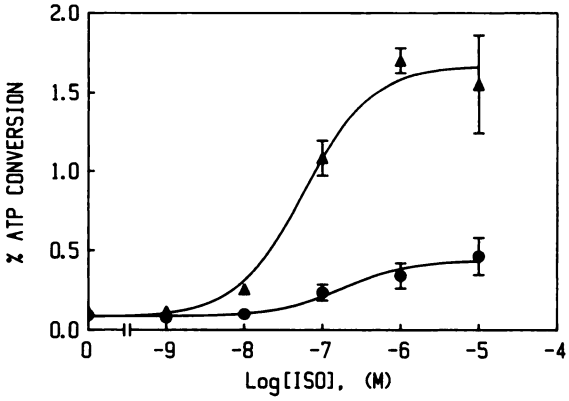

Fig. 3. Dose-response curves for ISO in 0.45 mM $CaCl_2$ in the absence (▲) and presence (●) of Bay K-8644. Results are from one of two experiments.

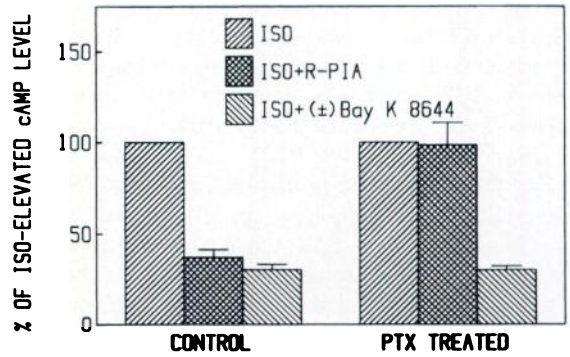

Fig. 4. Effects of (*R*)-PIA and Bay K-8644 on cAMP-elevating effect of ISO in control and pertussis toxin-treated cells (0.45 mM $CaCl_2$). Results are from one of two experiments.

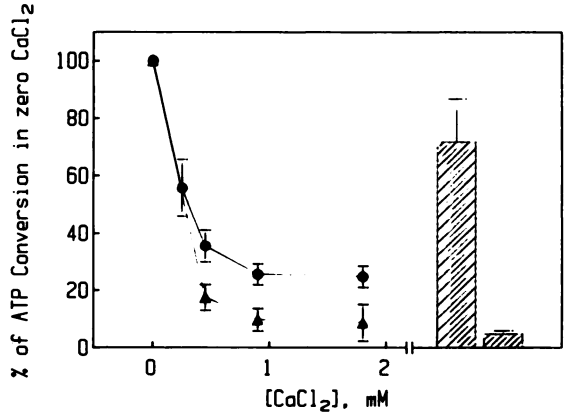

Fig. 5. Dose-response curves for $CaCl_2$ on the cAMP-elevating effect of (10 μM) ISO (▲) and (1 μM) forskolin (●). *Bar graphs*, response to ISO in the presence of 1.8 mM $CaCl_2$ plus 1 μM nifedipine (*left*) or Bay K-8644 (*right*). Results are from one of two experiments.

were not dependent on the presence of a low extracellular Ca^{2+} concentration. Nifedipine caused a large increase in the cAMP level in the presence of 1.8 mM $CaCl_2$ plus ISO; the depressant effect of Bay K-8644 was not as evident with 1.8 mM $CaCl_2$, because the cAMP level was already severely depressed. In an additional experiment cells were preincubated with or without nifedipine (1 μM) for 15 min before a 6-min exposure to 10 μM ISO (no PDE inhibitors). The percentage conversions measured in this experiment without PDE inhibitors were as follows: basal, 0.21 ± 0.01; nifedipine, 0.22 ± 0.02; ISO, 0.40 ± 0.01; ISO plus nifedipine, 1.3 ± 0.13 (six experiments). Thus, in the absence of PDE inhibitors ISO elevated cAMP approximately 2- and 6-fold in the absence and presence of nifedipine, respectively. Although the basal percentage conversion was not affected by nifedipine, this type of experiment cannot test possible relationships between Ca^{2+} entry via L-type calcium channels and cyclic variations in cAMP during the cardiac cycle, as reported by Brooker (20) more than 20 years ago.

With the exception of the experiment just discussed, all of the experiments described above were performed in the presence of two PDE inhibitors, rolipram and milrinone. Thus, although we have no evidence that the cardiac myocytes contain a Ca^{2+}/calmodulin-sensitive PDE, the presence of such an enzyme could explain our results. Because the available calmodulin inhibitors are rather nonspecific, producing effects on calcium channels (21), we did not believe that experiments with these agents would be informative. We did test the effects of D-600 and Bay K-8644 in the presence of a high concentration (0.5 mM) of the nonspecific PDE inhibitor IBMX. Although D-600 had no effect under these conditions and the effect of Bay K-8644 was blunted, the effect of ISO was not potentiated with respect to that in the presence of rolipram plus milrinone, as would be expected if the difference were due to the inhibition of a Ca^{2+}/calmodulin-sensitive PDE (data not shown). We therefore decided to further probe for an effect of calcium on cAMP catabolism by using a different approach. Specifically, we incubated [^{3}H]adenine-labeled cells with ISO (plus rolipram and milrinone) in 0.45 mM or 0 mM $CaCl_2$-containing medium for 6 min (a time by which the level of cAMP is at a steady state; data not shown), added the β receptor antagonist propranolol (10 μM), and measured cAMP levels at short time intervals thereafter (Fig. 6). We adjusted the concentration of ISO so that the cAMP levels at zero time (time propranolol was added) were similar, so that the rates of cAMP decrease could be validly compared. The $t_{1/2}$ values for the fall in cAMP were the same under the two conditions, giving additional evidence that the effects of Ca^{2+} concentrations are not on cAMP catabolism. In addition, a similar experiment (data not shown) demonstrated that the rate of cAMP fall in the presence of IBMX was identical to that in the presence of rolipram plus milrinone. This further suggests that the lack of an effect of D-600 on ISO-elevated cAMP levels in the presence of IBMX is not due to the inhibition of a Ca^{2+}/calmodulin-sensitive PDE.

The experiments presented thus far clearly show that extracellular calcium has a negative effect on the cAMP-elevating effect of ISO and that Ca^{2+} entrance through L-type calcium channels is necessary for this to occur. Although this effect appears to be at the level of adenylyl cyclase, rather than at the level of PDE, this is impossible to demonstrate directly in intact cells. We therefore determined the effects of Ca^{2+} on adenylyl cyclase activity in a crude membrane preparation of

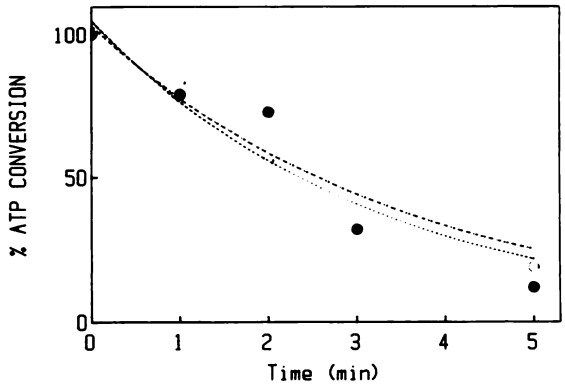

Fig. 6. Rate of change in cAMP levels in 0.45 mM (○, *dashed line*) and 0 mM (●, *solid line*) $CaCl_2$ after elevation by ISO and the subsequent blockade of β-adrenergic receptors by propranolol (10 μM). After a 6-min incubation with ISO (30 nM and 10 μM in 0 and 0.45 mM $CaCl_2$, respectively), propranolol was added (zero time) and the cAMP levels were determined at times thereafter. ATP conversion at zero time was set to 100%. The *curves* shown are the fits with a single-exponential equation. In all cases (three experiments, two conditions) the $t_{1/2}$ values were ~2 min.

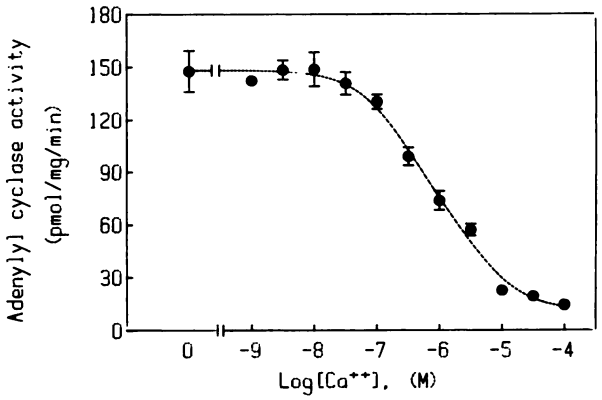

Fig. 7. Dose-response curve for the effect of Ca^{2+} concentration on adenylyl cyclase activity. The *line* drawn is for a two-site fit. Parameters are given in the text. Standard deviation bars are omitted where too small to show. Results from one of three experiments are shown.

myocyte membranes. The free Mg^{2+} concentration (0.8 mM) in these incubations was in the range of that reported for cytosolic free Mg^{2+} in embryonic chick myocytes (~0.5 mM) (22). The effect of Ca^{2+} on forskolin-stimulated activity was determined in these experiments, because receptor-mediated effects on adenylyl cyclase in chick myocyte membranes are poor (15), whereas the effect of forskolin is robust. Fig. 7 shows a dose-response curve for the effect of Ca^{2+} on forskolin-stimulated adenylyl cyclase activity. Adenylyl cyclase activity at 0.1 mM Ca^{2+} was reduced approximately 90%, compared with Ca^{2+}-free conditions. Although this curve was not markedly biphasic, as occurs in NCB-20 cell membranes (9) or canine sarcolemmal membranes (23), a marked inhibition at submicromolar Ca^{2+} concentrations was evident. The n_H for the curve in Fig. 7 is −0.74 (<1; $p < 0.05$). Although the two-site fit of this curve was not significantly better than the one-site fit ($p = 0.06$), the two-site analysis partitions the inhibition into approximately equal components with IC_{50} values of Ca^{2+} of approximately 0.3 and 4 μM. Additional data points (data not shown) demonstrated that the Ca^{2+} dose-response curve was unaffected by IBMX (0.5 mM). It is thus clear that Ca^{2+} inhibits adenylyl

cyclase at low concentrations characteristic of the inhibition of type V and VI adenylyl cyclase isozymes (8) and higher concentrations characteristic of the inhibition of all adenylyl cyclase isozymes (8).

There is considerable interest in "cross-talk" between different second messenger systems. It has been demonstrated in stable cell lines that agents that mobilize intracellular calcium via activation of the phospholipase C pathway can inhibit cAMP accumulation by directly inhibiting adenylyl cyclase activity (9–12). The present results clearly demonstrate that the cAMP-elevating effect of the β-adrenergic receptor agonist ISO in cardiac myocytes is blunted by a mechanism that involves the simultaneous increase in Ca^{2+} influx via L-type calcium channels. Although we have not measured $[Ca^{2+}]_i$ or determined the types of adenylyl cyclase in chick cardiac myocytes, our results strongly suggest that this feedback mechanism is based on a direct inhibitory effect of Ca^{2+} on one or more Ca^{2+}-sensitive adenylyl cyclase isozymes. Alternatively, indirect effects of Ca^{2+} could modify adenylyl cyclase activity. More experiments need to be done before our hypothesis is accepted or rejected. It is well established that the positive inotropic effect of catecholamines is mediated by the activation of adenylyl cyclase and a subsequent cAMP-mediated increase in calcium channel activity, The present results establish that this increase in calcium channel activity feeds back to blunt the effect of β receptor stimulation on adenylyl cyclase activity. The importance of this feedback loop in normal and pathological cardiac physiology and in the pharmacology of cardiotonic drugs remains to be determined.

Acknowledgments

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