BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 151–155 (1998)
ARTICLE NO. RC989626

Modulation of Cardiac Ca^{2+} Channels by IGF1

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Received October 2, 1998

Although there are several reports on the regulation of neuronal and skeletal muscle voltage-sensitive calcium channels by IGF1, the effects of short-term IGF1 exposure on cardiac Ca^{2+} channels have not been described. We measured the activity of nitrendipine-sensitive Ca^{2+} channels of intact cardiac myocytes in the presence of IGF1 by monitoring unidirectional Mn^{2+} influx measured as the quench of cytosolic fura-2 in electrically stimulated or K^+-depolarized cells. Maximal channel activation was observed after 10 min of preincubation with IGF1, which gave an increase of 216 +/− 25%. Treatment with the protein kinase C inhibitors bisindolylmaleimide I and chelerythrine mimicked the augmentation effect of IGF1, whereas PMA blocked enhancement of Mn^{2+} influx by IGF1. These results demonstrate that acute IGF1 augments dihydropyridine-sensitive sarcolemmal Ca^{2+} channel activity and that protein kinase C may contribute to the regulation of cardiac Ca^{2+} channels by IGF1.

Insulin-like growth factor has been identified recently as a regulator of cardiac growth and function (1,2,3,4,5,6). Recently, it has been found that administration of IGF1 by itself or in combination with growth hormone has beneficial effects on cardiac recovery following experimentally-induced trauma (4,18) and will enhance cardiac performance of the normal rat heart (7,19). In mice that have been genetically altered to reduce IGF1 expression, the defect resulted in an elevation in blood pressure and an increase in cardiac contractility of the left ventricle (10). Following myocardial infarction (5,8) or experimental-induced short-term artery constriction (6), both mRNA encoding IGF1 and IGF1 receptor are up-regulated in the heart. These results suggested that IGF1 and its receptor are coordinately expressed during cardiac trauma, and this may result in ventricular remodeling of infarcted cardiac tissue. Acute exposure to IGF1 and insulin results in enhanced rat ventricular papillary muscle cell contractility and associated $[Ca^{2+}]_i$ transients (9). This demonstrates that IGF and insulin may function as inotropic agents that alter intracellular Ca^{2+} handling, although the mechanism by which these agents modulate the $[Ca^{2+}]_i$ transients has not been determined (9).

Short-term administration of IGF1 to CNS neurons and neuronal cell-lines directly modulates voltage-activated calcium channel activity (11,12,13,14). In clonal pituitary cells, GH4C, acute IGF1 exposure increased L-type Ca^{2+} currents and spontaneous membrane electrical activity (11). In rat pinealocytes, L-type currents are inhibited by IGF1 and insulin (12); the tyrosine kinase inhibitor lavendustin A and the protein kinase C inhibitor calphostin C both blocked the effects of IGF1 and insulin, although the PI3 kinase inhibitor wortmannin was ineffective (12). In cerebellar granule neurons, IGF1 rapidly induced a large tyrosine kinase-dependent increase in L-type and N-type calcium channel currents. Overexpression of PI3 kinase and dominant negative PI3 kinase subunits, as well as application of the PI3 kinase inhibitors LY294002 and wortmannin reveal a requirement for PI3 kinase in this signaling pathway (13). In NG108 neuronal X glial hybrid cell-line, IGF1 has been shown to increase Ca^{2+} current density (L-type) and this was blocked by coincubation with the protein kinase C inhibitors, H7 and staurosporin (14). In addition, the L-type Ca^{2+} channel in rat skeletal muscle was demonstrated to be modulated by IGF1 in young and middle-aged rats, but not in aged rats (15). IGF1 increased phosphorylation of the $\alpha 1_s$ subunit Ca^{2+} channel by 10-fold in skeletal muscle from these younger animals, and this was blocked by preincubation with tyrosine kinase inhibitor genistein and a protein kinase C inhibitor peptide. However, little is known about the effects of IGF1 on cardiac Ca^{2+} channels (9).

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0006-291X/98 $25.00

Recently, Nicolas et al (25) have developed a single cell fluorescence imaging assay to measure dihydropyridine-sensitive cardiac Ca^{2+} channel activity by measuring Mn^{2+} influx through voltage-activated Ca^{2+} channels via the quench of the cytosolic fura-2 fluorescence intensity. We employed this assay to examine the regulation of dihydropyridine-sensitive cardiac Ca^{2+} channels by short term IGF1 in electrically stimulated and K^+-depolarized myocytes. We suggest that dihydropyridine-sensitive cardiac Ca^{2+} channels are modulated by acute IGF1 exposure, and that this depends on regulation by protein kinase C.

MATERIALS AND METHODS

Materials. IGF1 (recombinant human) was purchased from R&D Systems (Minneapolis, MN). All chemicals used to prepare buffers for cardiomyocytes were purchased from Sigma (St. Louis, MO). Bovine Serum Albumin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Collagenase was purchased from Worthington Biochemicals (Freehold, NJ). The fluorescent dye fura-2 was purchased from Molecular Probes (Eugene, OR). Phorbol 12,13-dibutyrate and bisindolylmaleimide I were purchased from Calbiochem (La Jolla, CA). H-89 and LY294002 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Chelerythrine HCl and Gö6976 were purchased from Alexis Biochemicals (San Diego, Ca).

Cell isolation. Calcium tolerant quiescent cardiomyocytes were isolated from the hearts of Sprague Dawley male rats (350-450 g) by perfusion as described previously (24). The animals were anesthetized prior to the isolation of the heart. The cells were suspended to a final concentration of 10^7 cells/ml in incubation buffer composed of 121 mM NaCl, 10 mM HEPES, 5 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM glucose and 1 mg/ml bovine serum albumin (BSA) at pH 7.4. After individual myocytes were isolated, their integrity was determined by microscopic examination. From 1.0g of ventricular tissue, approximately 10^7 cells were isolated. Approximately 65-70% had normal rod shaped morphology and integrity of the internal sarcomere structure as observed by light microscopy. For experiments measuring sarcolemmal Ca^{2+} channel activity the cardiomyocytes were loaded with fura-2 acetoxymethyl ester (5 μM) by incubation at room temperature for 25 min. with gentle shaking. Measurements carried out with cell populations have indicated that this protocol yields an average intracellular fura-2 concentration of 20-60 μM (24). The selected dye concentration was demonstrated previously in this laboratory to have no significant buffering effect on the cardiomyocyte contraction.

Measurement of calcium channel activity. Freshly isolated quiescent cardiomyocytes preloaded with fura-2/AM (5 μM) were placed on a laminin-coated (10 μg/ ml) and polylysine-coated (0.1 mg/ml) coverslip, mounted in the open flow chamber of the epifluorescence microscope. Cells were perfused continuously at a flow rate of 7 ml/min with an incubation buffer (130 mM NaCl, 4.7 mM KCl, 0.1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES/NaOH, pH 7.4) at room temperature (25°C). After 5 min perfusion with continuous electrical stimulation (1 Hz), the perfusion was switched to a buffer supplemented with 0.2 mM $MnCl_2$ and the electrical stimulation was stopped (25). Fluorescence images were collected every 2.50 s using 360 nm excitation and 510 nm emission filters. After 30 s during which the basal quench rate was determined, electrical stimulation (1 Hz) was initiated to activate Mn^{2+} quench of fura-2 via voltage sensitive Ca^{2+} channels. Blockage of voltage sensitive Ca^2 channels with the antagonist nitrendipine (10 μM) inhibited electrically-activated Mn^{2+} quench of cytosolic fura-2 by 95%. For pharmacological experiments, drugs were preincubated with myocytes for the designated incubation periods (see Results) before introduction of Mn^{2+} to the incubation buffer. Final outcome of the time course, dose response and drug studies are the cumulative results of at least 3 separate experiments using 11-29 cells for each experimental condition. The drug bisindolylmaleimide I (0.5 μM) used to examine the role of protein kinase C caused modest quenching of the fura-2 emission signal (510 nm), which resulted in an approximate 8-10% loss of signal intensity. For each experiment the fluorescence intensity of the cell of interest was normalized to the initial maximum signal intensity (360 nm), therefore, no correction was made for the quenching effect of this drug.

Statistics. Results are expressed as mean +/− S.E.M for the drug study analysis. Since the Mn^{2+} quench experiments employ separate cells tested before and after IGF1 and drug exposure, differences between means were analyzed by ANOVA. Analyses with $P < 0.05$ were considered statistically significant.

RESULTS

Recently reported by Freestone et al., acute exposure to IGF1 and insulin results in enhanced rat ventricular papillary muscle cell contractility and associated Ca^{2+} transients (9). In agreement with their findings, we found the electrical stimulation-induced cytosolic Ca^{2+} transient rate of rise and Ca^{2+} peak to increase after 5 min IGF1 (20 ng/ml) exposure in indo-1-loaded adult rat cardiomyocytes (28). The rate of rise of the Ca^{2+} transient was increased to 163% +/− 22% of control, and the peak rise (systolic ratio change from baseline) was increased to 141% +/− 16% compared to before IGF1 treatment. Previous results demonstrate that L-type Ca^{2+} channels in neurons (11,12) and skeletal muscle (15) are activated by IGF1, although there are no reports pertaining to the effects of IGF1 on cardiac Ca^{2+} channel activity. The approach we used to measure L-type Ca^{2+} channel activity in cardiomyocytes involves Mn^{2+} quench of cytosolic fura-2 in myocytes loaded with this dye (25). Activation of these channels by depolarization with electrical stimulation or 50 mM KCl results in influx of Mn^{2+}, and essential quenching of fura-2 due to the much stronger affinity for Mn^{2+} (K_d = 3 nM for Mn^{2+}, K_d = 250 nM for Ca^{2+} at physiological salt solution). Fura-2 quenching is measured at the Ca^{2+}-insensitive wavelength (360 nm). Although this approach is not as direct as whole-cell patch-clamp measurements of Ca^{2+} currents, it has the advantage of avoiding intracellular dialysis and consequent rundown of currents. We have used this method successfully to study the effects of PKC in cardiomyocytes, where the problems of Ca^{2+} channel rundown precludes direct Ca^{2+} current measurements.

Figure 1 is an actual trace of representative myocytes with and without IGF1 treatment (20 ng/ml, 5 min.). The passive rate of Mn^{2+} influx was first determined in quiescent myocytes exposed to 200 μM $MnCl_2$. Electrical stimulation was then applied (1.0 Hz, 5 ms pulse duration) and the rate of Mn^{2+} quench of cytosolic fura-2 was determined in cells pretreated in

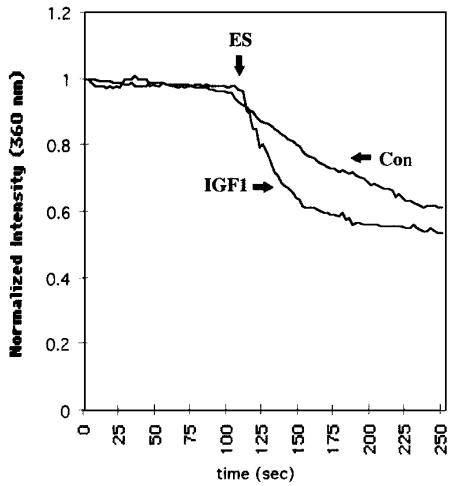

FIG. 1. Sarcolemmal Ca^{2+} channel activity measured by Mn^{2+} quench of cytosolic fura-2 in electrically stimulated myocytes. The graph illustrates the Mn^{2+} quench assay using representative traces of individual control and IGF1-treated (10 min, 20 ng/ml) myocytes. After Mn^{2+} addition, the basal rate of quench (360 nm excitation) was recorded for 30 s, followed by electrical stimulation (ES) (1 Hz, 5 ms pulse duration), which resulted in activation of Ca^{2+} channels and influx of Mn^{2+}.

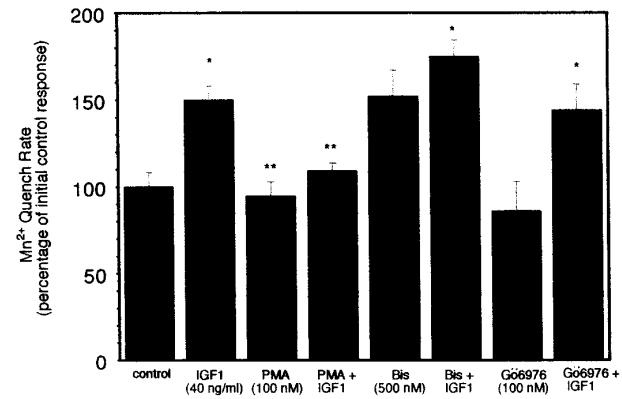

FIG. 3. Evaluation of the effects of protein kinase C modulators on IGF1 enhancement of Mn^{2+} influx. The rate of Mn^{2+} influx was determined by its quenching of cytosolic fura-2 in electrically stimulated myocytes before and after preincubation with PMA (100 nM, 10 min.), bisindolylmaleimide (0.5 μM, 10 min.), Gö6976 (0.5 μM, 10 min) and IGF1 (40 ng/ml, 5 min. following the drug incubation period). Mn^{2+} quench rates are expressed as a percentage of the control rate measured in cells prior to incubation with IGF1 or PKC modulators. Error bars indicate the s.e.m. determined from the average of 11-29 individual myocytes in at least 3 separate experiments. * represents statistically significant differences ($P < 0.05$) from the control cells response based on ANOVA analysis. ** represents statistically significant differences ($P < 0.05$) from IGF-treated cells response.

the presence or absence of IGF1. IGF1 augmented the rate of Mn^{2+} influx through sarcolemmal Ca^{2+} channels. Mn^{2+} influx was blocked by preincubation with 10 μM nitrendipine, demonstrating that Mn^{2+} entry is predominantly due to influx through L-type Ca^{2+} channels. Figure 2 shows the time course of the effects of IGF1 on Mn^{2+} pretreatment. After 10 min preincubation with the growth factor (20 ng/ml) IGF1 effects were maximal, and there was reduction in the maximal activation by IGF1 after 30 min preincubation (results from at least 3 separate experiments for each time point, 11-18 cells for each time point). It may be that the acute effects of IGF1 are transient. Preincubation

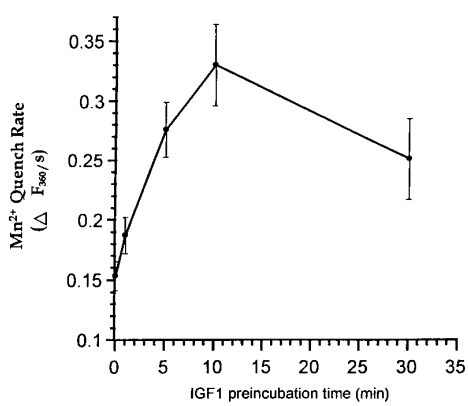

FIG. 2. Time course of IGF1 effects on Mn^{2+} influx. The rate of Mn^{2+} quench of cytosolic fura-2 in electrically stimulated myocytes was determined as shown in fig.1 after 0, 1, 5, 10 and 30 minutes preincubation with IGF1 (20 ng/ml).

with genistein (25 μM for 15 minutes) fully blocked IGF1 effects on electrically-stimulated myocytes, suggestion that tyrosine kinase domain of the IGF1 receptor is required for IGF1 to augment Ca^{2+} channel activity. However, genistein (25 μm) and its inactive analogue diadzein (25 μm) did not have inhibitory effects on control Mn^{2+} influx. In order to demonstrate that the enhancement of Ca^{2+} channel activity is not indirectly due to IGF1 modulation of sarcolemmal Na^+ channels and/or K^+ channels, 50 mM KCl-containing medium was used to depolarize the membrane and activate sarcolemmal Ca^{2+} channels directly. Preincubation with IGF1 (20 ng/ml, 5 minutes) resulted in an increase in Mn^{2+} influx rate by 160% +/− 10.1% (results from 14 and 16 cells from 3 separate experiments). Preincubation with nitrendipine (10μM) completely blocked KCl-induced Mn^{2+} influx.

Figures 3 and 4 show the results of a series of pharmacological experiments to evaluate the mechanism of IGF1 action in the signaling pathway responsible for augmenting Ca^{2+} channel activity. The effects of protein kinase C modulators are illustrated in figure 3. Pretreatment with 100 nM PMA for 10 min in the absence of IGF1 had a slight inhibitory effect on Ca^{2+} channel activity (94.6% of control). Pretreatment with PMA 5 min before exposure to IGF1 (40 ng/ml) significantly blocked the effects of IGF1 on Mn^{2+} influx rate (109% compared to 155% for IGF1-treated cells in this series of experiments). In addition, pretreatment with the protein kinase C inhibitors bisindolylmaleimide I

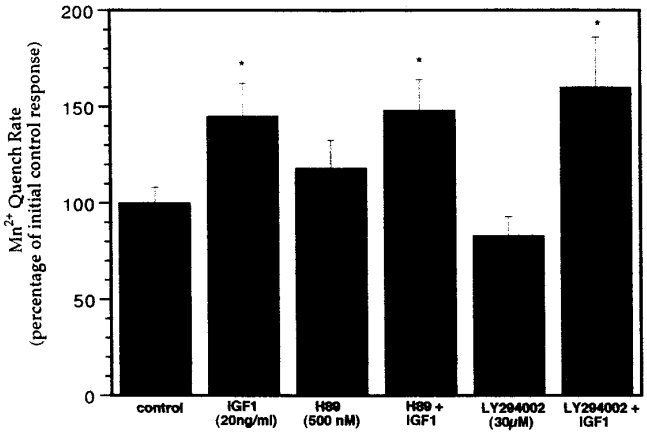

FIG. 4. Evaluation of the effects of protein kinase A and PI3 kinase inhibition on IGF1 augmentation of Mn^{2+} influx. The rate of Mn^{2+} quench of cytosolic fura-2 in electrically stimulated myocytes was determined after preincubation with H89 (0.5 μM, 10 min.), LY294002 (30 μM, 20 min.) and IGF1 (20 ng/ml, 5 min. following drug incubation period). Mn^{2+} quench rates are expressed as a percentage of the control rate measured in cells prior to incubation with IGF1 or PKA modulators. Error bars indicate the s.e.m. determined from the average of 7-29 individual myocytes in at least 2 separate experiments. * represents statistically significant differences ($P < 0.05$) from control cell responses based on ANOVA analysis.

($0.5\ \mu$M, 15 minutes) and chelerythrine (5 μM, 10 min, data not shown) appeared to mimic the effects of IGF1. The increased activity in cells pretreated with bisindolylmaleimide (152%) and IGF1 (158%) alone was only partially additive (175%), suggesting that inhibition of protein kinase C can mimic the effects of IGF1 on Mn^{2+} influx. Preexposure to the PKC inhibitor chelerythrine also increased Mn^{2+} influx rate to 145% of control. These results provide evidence that protein kinase C may play a role in the activation of sarcolemmal Ca^{2+}channels by IGF1. However, treatment with the classical PKC inhibitor, Gö6976 (0.5 μM, 10 min), caused no statistically significant difference from control on the rate of Mn^{2+} influx and did not alter the effects of IGF1 on the rate of Mn^{2+} influx. Figure 4 shows that treatment with the protein kinase A inhibitor H89 (0.5 μM, 10 min) did not block IGF1-induced augmentation of Mn^{2+} influx, although this drug effectively reduced isoproterenol (100 nM) augmentation of Mn^{2+} influx. Before preincubation with H89, isoproterenol augmented the rate of Mn^{2+} influx to 250% +/− 53%. After preincubation with H89, the effects of isoproterenol were reduced to 148% +/− 15% of control. In addition, the PI3 kinase inhibitor LY294002 (30 μM, 15 min.) did not block the effects of IGF1. Thus, in contrast to the IGF1 modulation of L-type Ca^{2+} channels in cerebellar granule neurons, which involves PI3 kinase (13), the PI3 kinase signaling pathway does not appear to be required for IGF1 modulation of cardiac L-type Ca^{2+} channels.

DISCUSSION

To examine the effects of short-term IGF1 on cardiomyocyte Ca^{2+} handling, we examined how IGF1 affected dihydropyridine-sensitive cardiac Ca^{2+} channels. We found that cardiac Ca^{2+} channel activity was augmented by IGF1, as measured by an increase in Mn^{2+} influx during membrane depolarization. Pretreatment with PMA blocked the enhancement of the rate of Mn^{2+} influx by IGF1 and pretreatment with the broad-range PKC inhibitors, bisindolylmaleimide I and chelerythrine, mimicked IGF1's effects. However, the classical PKC inhibitor, Gö6976, had no effect. Since the IGF1 response was not modulated by Gö6976, it is likely that the classical PKC family, including PKC α,β and γ, is not involved. Also, since pretreatment with PMA blocked the effects of IGF1, the atypical protein kinase C isozymes, PKCζ and PKCλ are probably not involved since they are insensitive to phorbol esters. Therefore, the novel PKCs δ, ϵ and η, which have previously been reported to be present in adult rat cardiomyocytes (26), are most likely involved with the regulation of cardiac Ca^{2+} channels by IGF1. The novel PKC family is sensitive to diacylglycerol and PMA, inhibited by bisindolylmaleimide I and chelerythrine and insensitive to Ca^{2+} and the classical PKC inhibitor, Gö6976.

Little is known about the role of protein kinase C in cardiac responses to IGF1, although in 3T3 mouse fibroblasts, acute exposure to IGF1[16] and other growth factors[17] causes translocation of PKC to the nucleus. Also, there are reports that IGF affects neuronal Ca^{2+} channel activity via a protein kinase C -dependent pathway (12,14). The cardiac Ca^{2+} channel has been found to be modulated by protein kinase C. In Xenopus oocytes, the cardiac Ca^{2+} channel α1 subunit by itself and in combination with the α2/δ, β and γ subunits was demonstrated to have a biphasic response to the protein kinase C regulator, PMA. Initially, PMA exposure resulted in augmentation of the expressed Ca^{2+} currents, but after 30 min the Ca^{2+} current decreased (27). Cardiac Ca^{2+} currents of neonatal rat cardiomyocytes also had a biphasic response to protein kinase C activation (23). Using both $^{45}Ca^{2+}$ influx and voltage clamp approaches, Lacerda et al. (23) demonstrated that 5 s PMA treatment resulted in an increase in Ca^{2+} current, whereas after 20 min exposure the effects were reversed with loss of Ca^{2+} current. These authors suggested that this biphasic response may be due to down-regulation of protein kinase C during the longer PMA incubation period. Recently, the cardiac L-type Ca^{2+} channel has been found to be modulated by PKC at the N-terminus, and it is at this region that the β2 subunit is known to interact. PMA treatment of oocytes overexpressing the cardiac Ca^{2+} channel α1 subunit results in an initial amplification of the Ca^{2+} current followed by a reduction in current (29). Deleting the

first 42 amino acids of this region results in reduced PMA responsiveness to current amplification, but does not effect the decrease in Ca^{2+} current. Coexpression with $\beta 2$ reduces the amplification of current by PMA, although there is no effect on the second half of this biphasic response. We found that 10 min PMA exposure slightly decreased cardiac Ca^{2+} channel activity, whereas, bisindolylmaleimide and chelerythrine treatment resulted in an increase in Ca^{2+} channel activity (see also Ref. 25). It may be that the Mn^{2+} quench assay measures the second part of the biphasic response reported by Shistik et al. (29). In contrast to rat myocytes, PMA and peptide inhibitors of protein kinase C had no effect on basal Ca^{2+} current of frog cardiac myocytes (20).

In the present study, we found that Mn^{2+} influx through nitrendipine-sensitive Ca^{2+} channels was slightly reduced by treatment for 10 min with PMA, and this treatment effectively blocked augmentation of Mn^{2+} influx by IGF1. It may be that a part of the reduction in electrically-triggered Mn^{2+} influx by PMA is due to its effects on other components of excitation -contraction coupling. Activators of protein kinase C have been shown to enhance the delayed-rectifier potassium current (22). In our system for measuring Ca^{2+} channel activity, PMA treatment fully blocked IGF1 augmentation of Mn^{2+} influx through voltage-dependent Ca^{2+} channels (L-type). The fact that the effects of IGF1 were still present in K^{+}-depolarized cells, which directly activates L-type Ca^{2+} channels, suggests an effect of IGF1 on the Ca^{2+}channel. Treatment with bisindolylmaleimide and chelerythrine resulted in an increase in Mn^{2+} influx, and the effects of bisindolymaleimide and IGF1 were not additive, indicating that suppression of PKC activity may mimic IGF effects. Therefore, we suggest a novel pathway whereby acute IGF1 exposure results in augmentation of cardiac Ca^{2+} channel activity via suppression of PMA-sensitive, Gö6976-insensitive protein kinase C activity. The increased activity of cardiac Ca^{2+} channels by IGF1 may contribute to enhancing cardiac muscle contractility (7,9) and the cytosolic $[Ca^{2+}]_i$ transients (9).

ACKNOWLEDGMENTS

We thank Dr. Dominique Rooney for her guidance and expertise in the design and execution of the research and also extend special thanks to Dr. Josep Nicolas for developing the manganese quench approach. This work was supported by National Institutes of Health Grants K01-AA00237 (to M.L.S.) and AA-07186 (to A.P.T.)

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