Crosstalk between Alpha-1A and Alpha-1B Adrenoceptors in Neonatal Rat Myocardium: Implications in Cardiac Hypertrophy

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ABSTRACT

The myocardial effects of alpha-1A adrenoceptor (alpha-1 AR) agonists in neonatal rats are mediated by alpha-1A AR and not by alpha-1B AR, although both receptor subtypes are equally expressed; the functions of alpha-1B AR are not known. Here, we report that alpha-1B ARs inhibit the activities of alpha-1A ARs in neonatal rat myocardium so that the inactivation of alpha-1B ARs by chloroethylclonidine (CEC) potentiated the effects of nonselective alpha-1 AR agonist phenylephrine (PE) on myocardial protein synthesis and early gene (c-fos and c-jun) expression. CEC did not modify the hypertrophic effect of angiotensin II. The potentiation of the effects of PE by CEC was associated with a translocation of Ca$^{2+}$-dependent protein kinase C (PKC)$\alpha$, which did not occur in the absence of CEC. Alpha-1A AR-selective agonist A61603 was ~1000-fold more potent than PE as a positive inotropic agent; it caused the translocation of PKC$\alpha$, which was not affected by CEC.

5-Methylurapidil antagonized the effects of PE and A61603, suggesting that these were mediated via alpha-1A ARs. Alpha-1D AR antagonist BMY 7378 did not modify PE-induced translocation of PKC. CEC potentiated the effects of PE on Ca$^{2+}$ transients in Fura 2-AM-loaded dispersed cardiomyocytes, and this potentiation was prevented by nifedipine. In whole-cell patch-clamp recordings of cultured cardiomyocytes, CEC potentiated the effect of norepinephrine on Ca$^{2+}$ channel currents, which was blocked by 5-methylurapidil. We conclude that alpha-1A ARs are positively and alpha-1B ARs are negatively coupled to nifedipine-sensitive Ca$^{2+}$ channels, possibly via G protein, and this antagonistic relationship between alpha-A AR and alpha-1B AR in the neonatal heart might be required physiologically for normal alpha-1 AR-mediated responses and myocardial development.

Alpha-1 ARs are G protein-coupled receptors and include alpha-1A AR, alpha-1B AR and alpha-1D AR corresponding to the cloned alpha-1a, alpha-1b and alpha-1d subtypes, respectively (Hieble et al., 1995). Alpha-1 ARs modify myocardial contractility, electrophysiological properties, metabolism and growth (Terzic et al., 1993). The effects of alpha-1 AR agonists on myocardial contractions and PI turnover are mediated by all the three alpha-1 AR subtypes in adult rats (Deng et al., 1996a, 1996b). Also, overexpression of cardiac-specific alpha-1B ARs in transgenic mice leads to cardiac hypertrophy as assessed at 10 weeks of age (Milano et al., 1994).

The expression and functions of alpha-1 AR subtypes appear to be developmentally regulated. Alpha-1D ARs cannot be detected in neonatal rat myocardium (Deng and Varma, 1997). However, 50% of the total alpha-1 ARs in neonatal rat hearts belong to alpha-1B AR subtype (Deng et al., 1996a), but the effects of alpha-1 AR agonists on cardiac contractions and PI turnover (Deng et al., 1996b), electrophysiology (del Balzo et al., 1990), hypertrophy (Knowlton et al., 1993) and calcium channel currents (Liu et al., 1994) in neonatal rats are different from those in adult rats (Milano et al., 1994). The expression and functions of alpha-1 AR subtypes appear to be developmentally regulated. Alpha-1D ARs cannot be detected in neonatal rat myocardium (Deng and Varma, 1997).

abbreviations: AR, adrenoceptor; Ang II, angiotensin II; BSA, bovine serum albumin; CEC, chloroethylclonidine; PI, phosphatidylinositol; 5 MU, 5-methylurapidil; NE, norepinephrine; PE, phenylephrine; PKC, protein kinase C; Prp, propranolol.
dent PKCα, Ca$^{2+}$-independent PKCe and PKC5, as well as atypical PKCζ (Clerk et al., 1994; Graham et al., 1996; Puceat et al., 1994; Rybin and Steinberg, 1994). An important function of alpha-1 AR agonists of pathophysiological relevance is cardiac hypertrophy (Morgan and Baker, 1991; Simpson, 1985). Neonatal rat cardiomyocytes in culture have been extensively used to study hypertrophic responses to alpha-1 AR agonists (Knowlton et al., 1993; Simpson, 1985). The hypertrophic effect of alpha-1 AR agonists in neonatal rat cardiomyocytes results from increased protein synthesis and is associated with an increased expression of c-fos, c-jun and Egr-1 (Iwaki et al., 1990), several other early genes and fetal genes, increased RNA synthesis and increased protein/DNA ratios (Morgan and Baker, 1991; Terzie et al., 1993). There is evidence that various hypertrophic stimuli up-regulate alpha-1A AR mRNA but down-regulate alpha-1B AR mRNA (Rokosh et al., 1996).

The purpose of the present study was to explore the myocardial function of alpha-1B ARs in neonatal rats using cardiac hypertrophy as a marker of biological response. Data revealed a potentiation of the hypertrophic effects of nonselective alpha-1 AR agonists PE by the alpha-1B AR agonist CEC; because these data suggested an interaction between alpha-1A AR and alpha-1B AR, experiments were done to identify the possible mechanism of this interaction using ventricular myocardium from 1- to 7-day-old neonatal rats.

Materials and Methods

Animals. Neonatal (1–7 day old) rats (Charles River, St. Constant, Quebec, Canada) were used according to a protocol of the McGill University Animal Care Committee. Rats were decapitated; hearts were quickly removed and ventricles used for different experiments.

Chemicals. A61603 (N-[5-(4,5-dihydro-1H-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl)methanesulfonamide hydrobromide) was a gift from Dr. Arthur Hancock (Abbott Lab, Abbott Park, IL). The pure (+)-enantiomer of the dihydropyridine agonist SDZ 202-791 was a gift from Sanof (Dorval, Quebec, Canada). CEC and 5-MU were from Research Biochemicals (Natwick, MA). Phenyl-ephrine HCl, di-propranolol HCl, L-norepinephrine bitartarate, aprotonin, leupeptin and bromodeoxyuridine were from Sigma Chemical (St. Louis, MO). Collagenase A was from Boehringer-Mannheim (Montreal, Quebec, Canada). Anti-PKC antibodies and PKC assay kits were from Gibco (Burlington, Ontario, Canada). Fura 2-AM and $[^{32}]$P$ATP (3000 Ci/mmol) were from Amersham (Oakville, Ontario, Canada). All other high-purity chemicals were from Fisher (Montreal, Quebec, Canada).

Agonist and antagonist concentrations. In many studies, PE was used at a concentration of 10 $\mu$M, which was found to produce ~50% of the maximal [H]Prp incorporation by cardiomyocytes (fig. 1). Alpha-1A AR-selective agonist A61603 was used at a 10 nM concentration because its EC$_{50}$ value on rat vas deferens and prostate ranges between 6 and 20 nM (Knepper et al., 1995) and its positive inotropic EC$_{50}$ value on neonatal rat ventricular myocardium was determined to be 17.1 $\pm$ 3.8 nM (n = 7). Whenever CEC was used, with the exception of patch-clamp studies, tissues were exposed to 10 $\mu$M CEC for 20 min and then washed with appropriate buffers before adding agonists; this protocol has been used by others (Michel et al., 1994) to study the roles of alpha-1 AR subtypes in isotropic responses of rat myocardium, and we (Deng et al., 1996a) found that the exposure of adult rat ventricles to 10 $\mu$M CEC for 20 min caused maximal inhibition of the isotropic effects of PE although 10 $\mu$M CEC did not inhibit the effects of PE on contractions of neonatal rat myocardium (Deng et al., 1996a). BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazineyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride) was used at a concentration of 30 nM because its pK$_{B}$ (the negative log of K$_{B}$) values against PE on adult rat aorta and right ventricles were 9 $\pm$ 0.13 and 6.87 $\pm$ 0.1, respectively; BMY 7378 did not inhibit the inotropic effects of PE on neonatal rat myocardium (Deng et al., 1996b). 5 MU was used to block alpha-1A AR at a concentration of 0.1 $\mu$M because its pK$_{B}$ value against PE on neonatal rat ventricles was found to be 7.86 $\pm$ 0.14 (Deng et al., 1996a).

Inotropic responses to PE and A61603. Right ventricles from 6- to 7-day-old rats were divided into halves and set up at 32°C in tissue baths containing Krebs' buffer of the following composition (mM): NaC1 117, NaHCO$_{3}$ 25, KCl 4.7, CaCl$_{2}$ 1.8, MgSO$_{4}$ 1.5, KH$_{2}$PO$_{4}$ 1.1, dextrose 11 and EDTA 0.03. The buffer was equilibrated with a mixture of 95% O$_{2}$ and 5% CO$_{2}$ and preparations were stimulated at 1 Hz and 30 V. Isometric contractions were recorded by means of Grass force-displacement transducers on a Grass polygraph exactly as described before (Deng et al., 1996a, 1996b). After stabilization, 1 $\mu$M Prp was added to block beta ARs; 30 min later, one right ventricular piece was used to construct an inotropic concentration-response curve to PE, and the other was used to construct an inotropic concentration-response curve to A61603.

PKC activity. Whole right ventricles were set up in Krebs' buffer at 32°C exactly as described above for recording inotropic responses; in most studies, four tissues, each from separate 5- to 7-day-old rats from the same litter, were used at the same time. All preparations were exposed to 1 $\mu$M propranolol for 30 min before the addition of alpha-1 AR agonists. Time and concentration dependence for PKC translocation by PE was first determined. Because maximal PKC translocation occurred 5 min after adding PE, this period was selected in all subsequent studies designed to determine the effects of different treatments on PKC translocation. One preparation was first exposed to 10 $\mu$M CEC for 20 min followed by a washout. Prp (1 $\mu$M) was added to all preparations, and 30 min later, alpha-1 AR agonists were added; 5 min after the addition of alpha-1 AR agonists, tissues were quickly removed and immediately homogenized in 2 ml of ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA and 0.5 mM EGTA) containing 25 $\mu$g/ml leupeptin, 25 $\mu$g/ml aprotinin, 10 mM 8-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride by means of a precooled Polytron homogenizer (5-sec pulses at setting 7; Brinkmann Instruments, Westbury, NY) in the cold room. The homogenates were centrifuged at 105,000 $\times$ g for 60 min. The supernatant constituted the soluble fraction; the pellets were suspended in 2 ml of buffer A plus 1% Triton X-100 and left on ice for 20 min and centrifuged again at 105,000 $\times$ g for 60 min. The resulting supernatant was saved as the particulate fraction. Both fractions were applied to DEAE columns (Whatman) equilibrated with buffer B (20 mM Tris, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 8-mercaptoethanol). PKC was eluted with 1.5 ml of buffer B containing 0.2 M NaCl. The PKC activity was determined by a commercial PKC assay kit (GIBCO).

Western analysis of PKC isoforms. Partially purified myocardial particulate and soluble fraction proteins (6–8 $\mu$g) were subjected to SDS-PAGE using 10% SDS-polyacrylamide gels. The proteins were transferred from the gels to nitrocellulose membranes at 250 mA for 90 min. The membranes were blocked for 1 h at 37°C in 2.5% skimmed-milk powder in 0.1% Tween in Tris-base saline (TTBS) and probed overnight with antibodies against specific PKC isoforms at 4°C; the membranes were washed with TTBS and then incubated with the peroxidase-linked second antibody, and the immunoreactive bands were visualized by using the enhanced chemicoluminescence kit (Amersham).

Cardiomyocyte dispersal. Ventricles from 1- to 3-day-old rats were dissociated by collagenase treatment and plated in Petri dishes for 90 min. Unattached cells (primarily cardiomyocytes) were incubated in modified Joklik MEM containing 0.1% BSA and 1 mM CaCl$_{2}$ for 6 hr, after which cardiomyocytes were used either for measuring
Ca⁺⁺ transients or were cultured to study [3H]Phe incorporation, c-fos and c-jun expression and Ca⁺⁺ channel currents. 

[3H]Phe incorporation. Dispersed cardiomyocytes were plated onto Primaria culture dishes (Falcon); ~300,000 cells/well were incubated in RPMI 1640 medium for 24 hr in the presence of 0.1 mM bromodeoxyuridine (Simpson, 1985). Cardiomyocytes cultured for 24 hr in serum-free medium (48 hr after cell dispersal) were used to study the effect of PE on [3H]Phe incorporation. For this purpose, cells were incubated for 24 hr with 1 μCi/ml [3H]Phe (60 Ci/mmol) alone or in the presence of increasing concentrations of PE. Because 10 μM PE produced ~50% of the maximal increase in [3H]Phe incorporation, the modifications of the response to 10 μM PE by 0.1 μM 5 MU or 10 μM CEC were determined; for this purpose, PE was added in the presence of 0.1 μM 5 MU or cells were first exposed to 10 μM CEC for 20 min and then washed with serum-free media before the addition of [3H]Phe and PE. After the end of the 24-hr incubation, cells were washed four times with serum-free medium and solubilized in 0.1 N NaOH, and the total radioactivity counted. Total proteins were measured by the dye-binding method using BSA as the standard.

Northern analysis of c-fos and c-jun. Total RNAs of cardiomyocytes exposed to different agents were extracted by the Trizol method (GIBCO); RNAs were quantified by absorbance at 260 nm, and their integrity was verified by formaldehyde-agarose gel electrophoresis followed by ethidium bromide staining. For slot blotting, 20-μg aliquots of total RNAs were denatured in a buffer (pH 7.5) composed of 80% deionized formamide, 1 M formaldehyde and 5× SSC (1× SSC was 15 mM NaCl, 1.5 mM sodium citrate) at 90°C for 5 min and applied to nylon membranes under vacuum using a commercial slot blotting apparatus. After crosslinking of nucleic acids to the membranes by UV, the membranes were incubated in hybridization solution [5× SSC, 5× Denhardt’s reagent (0.1%/w/v each of Ficoll-400, polyvinlypyrrolidone, BSA fraction V), 10% dextran sulfate, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA and 10⁶ cpm/ml of denatured radiolabeled c-fos, c-jun or β-actin probe] overnight at 60°C. cDNA fragments of mouse c-jun, mouse c-fos and rat β-actin (Ambion) were labeled with random labeling kit (Amersham) and [α-32P]dCTP (3000 Ci/mmole). After hybridization, the membranes were washed three times with 1× SSC containing 0.5% SDS at 60°C for 30 min each. The radioactive bands were detected and quantified by PhosphorImaging (Molecular Dynamics).

Cytosolic calcium transients. After a 6- to 8-hr incubation of dispersed cardiomyocytes, cells were loaded with 2 μM FURA 2 AM for 30 min with or without CEC (10 μM) in Hanks’ balanced salt solution and then gently spun at 23°C. After washing free Fura 2-AM and CEC, 2-ml aliquots of cell suspension containing 100,000 cardiomyocytes were placed into cuvettes. The fluorescence of the continuously-stirred whole-cell suspension was monitored in an excitation light source from a Spek dual-wavelength spectrophluorometer system. Excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were applied, and emission signals were digitized and stored in a microcomputer. 4-Bromo-A23187 (50 μM) and EGTA (5 mM) were added to obtain maximum and minimum fluorescence, respectively. The ratio of emitted light at 340 nm to 380 nm was calculated. The positive inotropic response to 10 μM PE was inhibited by 0.1 μM 5 MU but potentiated by 10 μM CEC (fig. 1b). CEC did not modify the hypertrophic activity of 100 nM Ang II (data not shown). Inactivation of alpha-1B ARs by CEC also potentiated PE-induced increase in the expression of myocardial c-fos and c-jun mRNAs (fig. 1c and d).

Translocation of PKC. PE caused a time- and concentration-dependent increase in particulate PKC activity in electrically stimulated neonatal rat ventricles; the maximal effect was observed at 5 min (fig. 2a) at 100 μM PE (fig. 2b). The increase in particulate PKC activity was not uniformly associated with a decrease in the soluble fraction. The translocation of PKC by 10 μM was inhibited by 0.1 μM 5 MU (fig. 2c). In contrast, inactivation of alpha-1B ARs by 10 μM CEC markedly potentiated (2.3-fold) the response to PE (fig. 2c); this potentiation was prevented by 1 μM nifedipine (fig. 2c), which did not modify the effect of PE in the absence of CEC. CEC did not modify the effects of 100 nM Ang II on PKC activation (data not shown). Alpha-1D AR antagonist BMY 7378 (30 nM) did not modify the effect of 10 μM PE on PKC translocation (data not shown).

At a concentration of 10 nM, A61603 caused a greater increase in particulate PKC activity than did 10 μM PE (fig. 2d); the effect of 10 nM A61603 was not potentiated by 10 μM CEC but was blocked by 0.1 μM 5 MU (fig. 2d).

Calcium transients. In freshly dispersed cardiomyocytes preloaded with Fura 2-AM, 10 μM PE increased calcium transients by 20% over the basal level (fig. 3). CEC (10 μM) potentiated and 5 MU (0.1 μM) inhibited PE (10 μM)-mediated increase in Ca⁺⁺ transients (fig. 3), which was completely inhibited by 1 μM nifedipine (data not shown).
Whole-cell recording of Ca\(^{2+}\) channel activity. Both T- and L-type Ca\(^{2+}\) channel currents in cultured cardiomyocytes measured in whole-cell configuration in the absence and the presence of different agents are illustrated in figure 4a. The changes in peak currents were elicited by a paired pulse to −20 and 0 mV (fig. 4b). Steady-state effects of CEC (10 µM), NE (5 µM), 5 MU (0.5 µM and 1 µM), Prp (1.5 µM) and (+)-SDZ 202–791 (5 µM) on the current kinetics are shown in figure 4c. The effects of the drugs were observed at or above −20 mV in 20 mM Ca\(^{2+}\) recording solution (fig. 4, d–g). Ca\(^{2+}\) currents were stable, and the addition of CEC (10 µM, n = 8) produced little measurable changes in peak currents (fig. 4a) or current-voltage relationship (fig. 4d); however, the subsequent addition of NE (5 µM, n = 8) after treatment with CEC caused a rapid and marked increase in Ca\(^{2+}\) currents to 200% of the control values (fig. 4, a, c and e). NE (5 µM, n = 4) produced only a modest increase in Ca\(^{2+}\) currents (fig. 4h), which was further enhanced by CEC. 5 MU (0.5 µM) reversed by 80% the CEC-induced increase in the effect of NE (n = 10, fig. 4); the remaining NE-stimulated current was reversed by 1.5 µM propranolol (n = 8). Neither NE (n = 6, not shown) nor a combination of NE and CEC (fig. 4e) exerted any effects on the T-type Ca\(^{2+}\) currents. The effect of NE plus CEC did not appear to be voltage dependent, nor did it exhibit the deactivation of the L-type Ca\(^{2+}\) currents, unlike the effect of dihydropyridine agonist SDZ 202–791 (fig. 4g). NE was used for the patch-clamp studies instead of PE because no consistent effect of PE on Ca\(^{2+}\) current was observed.

PKC isoforms. PE (10 µM) caused translocation of Ca\(^{2+}\)-independent PKC isoforms PKCe and PKCδ but exerted little effect on Ca\(^{2+}\)-dependent PKCa and atypical PKCγ (fig. 5a); PKCδ was not detected. CEC (10 µM) potentiated the PE-induced translocation of Ca\(^{2+}\)-dependent PKCa but not of other PKC isoforms (fig. 5b); this effect of CEC was inhibited by 1 µM nifedipine (fig. 5, a and b). In contrast to PE, 10 nM
A61603 caused the translocation of PKCα in the absence of CEC treatment (fig. 5, c and d).

Discussion

Alpha-1B ARs are abundantly expressed in neonatal rat myocardium (del Balzo et al., 1990; Deng et al., 1996a) but do not mediate any of the classic effects of alpha-1 AR agonists; because we have shown that these receptors contribute to the positive inotropic responses and PI turnover in adult rat myocardium (Deng et al., 1996a), it would appear that either alpha-1B ARs are not coupled to signal transduction pathways in neonatal period or they perform some unknown functions. The present study was performed to explore these possibilities.

The inference drawn in this study is based on the use of agonists and antagonists as tools, and significant conclusions are based on the assumption that the effects of CEC were the result of irreversible inhibition of alpha-1B ARs and little or no effect on alpha-1A ARs and alpha-1D ARs. CEC is known to irreversibly inactivate alpha-1B ARs (Minneman et al., 1988) and, to a lesser extent, alpha-1D ARs (Perez et al., 1991) but was found to exert little effect on alpha-1A ARs (Minneman et al., 1988). On the other hand, recent data (Hirasawa et al., 1997) suggest that the selectivity of CEC is dependent on its access to alpha-1 AR subtypes rather than any differences in the structure of alpha-1A ARs and alpha-1B ARs. Notwithstanding these data, our conclusions that the effects of CEC are due to the inactivation of alpha-1B ARs are justified for two reasons. First, in this study tissues were exposed to CEC for 20 min followed by washout before the addition of agonists, which should minimize effects on alpha-1A ARs (Deng et al., 1996a; Michel et al., 1994; Minneman et al., 1988). Second and more important, if CEC did inhibit alpha-1A ARs, it would be expected to inhibit the effects of PE as well as those of the alpha-1A AR-selective agent A61603; instead CEC potentiated the effects of PE and did not modify the effects of A61603, which is incompatible with a significant inhibitory effect of CEC on alpha-1A ARs under the experimental conditions of this study. Indeed, 5 MU, a relatively selective alpha-1A AR antagonist completely inhibited different effects of PE investigated in this study.

Because alpha-1D ARs are not functionally expressed in neonatal rat hearts (Deng and Varma, 1997) and are minimally expressed in hearts (Deng et al., 1996b; Yang et al., 1997) and most other tissues of adult rats (Yang et al., 1997) with the exception of aorta (Deng et al., 1996b), it was assumed that the effects of CEC treatment are not due to an inactivation of alpha-1D AR. This inference is supported by the observation that the alpha-1D AR antagonist BMY 73778 (Goetz et al., 1995) at a concentration of 30 nM, ~3 times its pKₐ value against PE on aorta (Deng et al., 1996b), did not modify the effect of PE on the translocation of PKC. It is thus reasonable to infer that changes in different myocardial responses to alpha-1 AR agonists after treatment with CEC were primarily due to an inactivation of alpha-1B AR and not due to inactivation of alpha-1A AR and alpha-1D AR.

PE increased [³H]Phe incorporation as well as c-fos and c-jun expression in neonatal rat cardiomyocytes; this effect of PE was inhibited by alpha-1A AR antagonist 5 MU and potentiated by alpha-1B AR antagonist CEC (fig. 1). It has been shown by other workers (Knowlton et al., 1993) that the cardiac hypertrophic effects of PE are mediated by alpha-1A AR; this conclusion is supported by the data of this study. However, the potentiation of the effects of PE on cardiac protein synthesis and gene (c-fos, c-jun) expression by CEC has not been previously reported; we interpret these data to imply that alpha-1B ARs exert an inhibitory effect on alpha-1A ARs so their inactivation by CEC leads to an increase in the effects of PE via alpha-1A ARs.

Our data suggest that the potentiation of the cardiac hypertrophic effect of PE by CEC results from an increase in the translocation of PKC. The inhibition of the PE-induced translocation of PKC to the plasma membrane (fig. 2) by 5 MU suggests that the effects of PE were mediated by alpha-1A ARs and coupled to DAG/PKC pathways. The potentiation of the effect of PE after inactivation of alpha-1B ARs by CEC suggests that alpha-1B ARs decrease the activities of alpha-1A ARs by inhibiting the agonist-mediated translocation of PKC; this inference is supported by the data that CEC alone did not exert any effects on PKC translocation. A61603 is a selective agonist for alpha-1A ARs (Knepper et al., 1995) whereas PE acts on both alpha-1A ARs and alpha-1B ARs (Tereziec et al, 1993). Because CEC potentiated the effects of PE but not of A61603 on PKC translocation (fig. 2, c and d), it would appear that the stimulation of alpha-1B AR is required for these receptors to inhibit the functions of alpha-1A AR. It might be added that almost 1000-fold higher potency of A61603 relative to PE in inotropic assay and an apparently higher efficacy of A61603 than of PE on the translocation of PKC might also support our inference that the myocardial effects of alpha-1 AR agonists are produced via alpha-1A AR and inhibited by alpha-1B AR.
The L-type Ca\(^{2+}\) channel blocker nifedipine (1 \(\mu M\)) did not modify the effect of PE on PKC activity. Because PE causes the translocation of Ca\(^{2+}\)-independent PKC isoforms (Deng et al., 1997) as also confirmed in the present study (fig. 5), it is not too surprising that nifedipine did not inhibit PE-induced translocation of PKC in the absence of CEC treatment. On the other hand, nifedipine abolished the potentiating effect of CEC on PE-induced PKC activation (fig. 2c). These results suggest that the inhibition of alpha-1A AR-mediated effects by alpha-1B AR is exerted via L-type Ca\(^{2+}\) channels. This inference is supported by two additional sets of data. First, the inactivation of alpha-1B AR by CEC potentiated the effects of PE on Ca\(^{2+}\) transients in dispersed cardiomyocytes (fig. 3). Second, NE-induced increase in Ca\(^{2+}\) channel current in cardiomyocytes, recorded by whole-cell patch-clamp, was potentiated by CEC and inhibited by 5 MU (fig. 4); these data also suggest that the interactions between alpha-1B AR subtypes of alpha-1A ARs and alpha-1B ARs are primarily because of an increase in membrane channel current rather than by a mobilization of intracellular Ca\(^{2+}\). The inhibition of a component of NE-induced increase in Ca\(^{2+}\) current by Prp (fig. 4) suggests involvement of beta ARs in the effects of NE on the Ca\(^{2+}\) channel current as demonstrated by other workers (Chen et al., 1996).

If our assumption that the inactivation of alpha-1B ARs potentiates the effect of alpha-1 AR agonists by increasing Ca\(^{2+}\) current is correct, one would expect an expression of this phenomenon on Ca\(^{2+}\)-dependent PKC isoforms; indeed, this seems to be the case. PE caused the translocation of Ca\(^{2+}\)-independent PKC isoforms delta and epsilon but exerted little effect on Ca\(^{2+}\)-dependent PKCa and on PKCz as previously reported (Deng et al., 1997). However, after the inactivation of alpha-1B AR by CEC, PE caused a marked translocation of Ca\(^{2+}\)-dependent PKCa isoform (fig. 5). These data suggest that alpha-1B AR activation inhibits the translocation of PKCa and its inactivation is necessary for its translocation by the nonselective alpha-1 AR agonist PE. This is consistent with the data that A61603, which activates alpha-1A AR but not alpha-1B AR (Knepper et al., 1995), caused translocation of PKCa even in the absence of CEC and the effect of A61603 was not modified by CEC.

Taken together, data of this study reveal an interesting interaction between two alpha-1 AR subtypes in neonatal myocardium with respect to cardiac hypertrophy. There is evidence of interactions between myocardial alpha-1 ARs and beta ARs (Chen et al., 1996; Oleksa et al., 1996; Varma, 1991). As well, various hypertrophic stimuli, including NE (Rokosh et al., 1996) and thyroid state (Lazar-Wesley et al., 1991), can differentially regulate alpha-1A ARs and alpha-1B ARs. However, our data for the first time present strong evidence for an antagonistic relationship between alpha-1A ARs and alpha-1B AR subtypes of alpha-1 ARs. The possible mechanism of the interaction between alpha-1A ARs and alpha-1B ARs is presented in the proposed model (fig. 6). According to this model, alpha-1A ARs are positively coupled to both nifedipine-sensitive Ca\(^{2+}\) channels and DAG/PKC pathways, whereas alpha-1B ARs are negatively coupled to Ca\(^{2+}\) channels, possibly via pertussis toxin-sensitive G protein (possibly G\(_{i}\), for which there is some evidence in the literature; Akhter et al., 1997) and uncoupled to DAG/PKC pathways. This model is consistent with the reported higher coupling efficiency of alpha-1A ARs than of alpha-1B ARs and alpha-1D ARs (Theroux et al., 1996). Our data do not exclude the possibility that the inactivation of alpha-1B ARs by CEC might alter the efficacy of alpha-1A ARs to alpha-1 A AR agonists; however, this inference is not favored by an
observed lack of potentiation of the effects of alpha-1A AR-selective agonist A61603 by CEC.

It is quite possible that such an antagonist relationship between alpha-1A ARs and alpha-1B ARs also exists in the adult. However, alpha-1B ARs seem to be positively coupled to intracellular signaling pathway because many of the myocardial effects of alpha-1 AR agonists (Terzic et al., 1993), including those on contractions (Deng et al., 1996a), can be inhibited by CEC. Also, the overexpression of cardiac-specific alpha-1B AR in transgenic leads to cardiac hypertrophy (Milano et al., 1994), which is produced only by alpha-1A ARs in neonatal rat cardiomyocytes (Knowlton et al., 1993). Thus positive coupling to intracellular signaling mechanism and negative coupling to Ca \(^{2+}\) channels might produce opposing effects, and the biological responses to alpha-1 AR agonists will depend on the relative roles of these two pathways. In contrast to the results in transgenic mice with overexpression of constitutively active alpha-1B ARs (Milano et al., 1994), the overexpression of wild-type alpha-1B ARs does not lead to cardiac hypertrophy but rather to an attenuation of cardiac responses to norepinephrine and PE (Akhter et al., 1997); interestingly, the attenuation of cardiac effects of alpha-1 AR agonists is reversed by pertussis toxin, which might imply that negative coupling of alpha-1B ARs to calcium channel via Gi protein was responsible for the decrease in alpha-1 AR-mediated effects. On the other hand, no significant change in heart weight-to-body weight ratio of adult transgenic mice deficient in alpha-1B ARs was observed (Cavalli et al., 1997), which might suggest that in the mouse, either a deficiency of alpha-1B ARs does not modify cardiac development or any abnormality that might be present during neonatal period does not persist as the animal matures.

In conclusion, this study demonstrates that alpha-1B ARs inhibit the functions of alpha-1A AR-selective agonist A61603 by CEC.

Fig. 6. Proposed model of the antagonistic relationship between alpha-1A ARs and alpha-1B ARs in the myocardium of neonatal rats. In this model, alpha-1B ARs are negatively coupled to nifedipine-sensitive Ca \(^{2+}\) channels, possibly via G protein, and uncoupled to diacylglycerol (DAG)/PKC pathways (dashed line), whereas alpha-1A ARs are positively coupled to both (solid line). Inactivation of alpha-1B AR results in unopposed activation of alpha-1A ARs by an increase in Ca \(^{2+}\) influx, activation of PKC and subsequent events leading to early gene expression and myocardial hypertrophy.

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