Pacioretty, Linda M., and Robert F. Gilmour, J. R.

Restoration of transient outward current by norepinephrine in cultured canine cardiac myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1599–H1605, 1998.—The mechanism for the reduction of the transient outward K⁺ current (Iₒ) in diseased myocardium is unknown. To identify potential mechanisms, the reduction of Iₒ and its subsequent restoration by norepinephrine (NE) were studied in cultured canine epicardial myocytes. After myocytes were cultured for 9 days (day 9), Iₒ density was decreased compared with density on the day of isolation (day 0) (3.2 ± 0.4 vs. 10.4 ± 0.4 pA/pF; mean ± SE). The time constant of current decay (τdecay) was increased, the time course of recovery from inactivation was prolonged, and the half-inactivation voltage (V½) was shifted to less negative potentials. Exposure of myocytes on day 8 to 1 µM NE or isoproterenol (Iso) for 1 h had no acute effect on Iₒ but restored Iₒ density to 7.6 ± 1.2 or 9.7 ± 2.3 pA/pF, respectively, on day 9. Recovery from inactivation and τdecay remained slowed, and V½ shifted to less negative potentials. The effects of NE and Iso were blocked by actinomycin D and were not mimicked by phenylephrine or phorbol ester. A-23187 (1 µM) also restored Iₒ. Thus β-adrenergic agonists restored normal Iₒ density, but not normal Iₒ kinetics, in cultured epicardial myocytes, possibly via increased intracellular Ca²⁺ concentration.

The density of the 4-aminopyridine (4-AP)-sensitive transient outward K⁺ current (Iₒ) typically is low in the neonatal heart and in hypertrophic and myopathic adult hearts (9, 13, 14, 17, 21, 23–25, 31). Although the mechanism for the reduction of Iₒ density in developing or diseased myocardium has not been established, several lines of evidence suggest that the expression of Iₒ in the developing heart is modulated by a trophic effect of the sympathetic nervous system and that the reduction of Iₒ in diseased myocardium is caused by the loss of that effect (9, 12, 13, 26). However, studies of the mechanisms responsible for the reduction of Iₒ in disease states such as Chagas’ disease (12, 23), X-linked muscular dystrophy (24), and inherited sudden death (9) have been hampered by the cost of maintaining colonies of affected animals and by variability between animals in the expression of their disease. To circumvent these limitations, we have in the present study exploited the observation by Schackow et al. (28) that Iₒ density decreases in adult feline ventricular myocytes with time in primary culture. Our expectation was that, by identifying the mechanism for the reduction of Iₒ in cultured myocytes, we would gain insights into potential mechanisms for the reduction of Iₒ in diseased myocardium.

The initial objective of our study was to determine whether the density of Iₒ is reduced in cultured adult canine ventricular myocytes as it is in cultured feline myocytes (28). Once it became apparent that Iₒ density was reduced progressively with time in culture, we tested whether Iₒ could be restored by exposure to norepinephrine (NE) and whether such a restorative effect was mediated by α- or β-adrenergic receptors. We also tested whether the restorative effect of NE on Iₒ was mediated by increased intracellular Ca²⁺ concentration.

METHODS

Epicardial myocyte isolation. Adult beagle dogs (n = 24) of either sex were obtained from a colony of inbred dogs maintained by Cornell University. The dogs were anesthetized with Fatal-Plus (390 mg/ml pentobarbital sodium, 0.2 mg/4.5 kg iv; Vortech Pharmaceuticals, Dearborn, MI). Hearts were removed rapidly via a left thoracotomy and placed in cold, oxygenated (95% O₂-5% CO₂) Tyrode solution containing (in mM) 0.7 MgCl₂, 0.9 NaH₂PO₄, 2.0 CaCl₂, 124 NaCl, 24 NaHCO₃, 4 KCl, and 5.5 glucose; pH 7.4. The circumflex coronary artery or a branch of the left anterior descending coronary artery was cannulated and a portion of the left ventricle excised. The tissue initially was perfused with Tyrode solution at 37°C. After 10–15 min, the perfusion was switched to a Ca²⁺-free solution containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 0.68 glutamine, 11 glucose, 25 NaHCO₃, 5 pyruvate, 2 mannitol, and 10 taurine; pH 7.3. At ~3–5 min, collagenase (0.4 mg/ml; type II, Worthington Biochemical) and BSA (0.5 mg/ml; Sigma) were added and perfusion was continued for another 10–12 min. Digested tissue was sliced away from the subepicardial area, placed in 10 ml of enzyme solution, and swirled. The supernatant was collected, and 10 ml of fresh Ca²⁺-free solution with 0.4 mg/ml collagenase and 0.5 mg/ml BSA was added to the slurry and gently bubbled in a water bath maintained at 37°C. Supernatant was collected for six subsequent washes. After 5 min of settling, the final pellet was washed in 10 ml of incubation buffer containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 0.68 glutamine, 11 glucose, 20 NaHCO₃, 5 pyruvate, 10 taurine, and 0.5 CaCl₂ plus 2% BSA. After another 30 min of settling, the pellet was washed a second time with incubation buffer now containing 1 mM CaCl₂ and was allowed to equilibrate at room temperature for 60 min.

All isolation solutions were filter sterilized and contained penicillin-streptomycin (100 U/ml penicillin-100 µg/ml streptomycin). The perfusion apparatus also was sterilized, and perfusion and subsequent bubblings were conducted in a laminar flow hood.

Epicardial myocyte culture. Once isolated, the myocytes were plated at a density of ~7 × 10³ cells/cm² on plastic culture dishes coated with laminin (10 µg/ml; GIBCO). The culture medium consisted of Eagle’s minimum essential H1599

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Fig. 1. Transient outward K⁺ current (Iₒ) activation in day 0 (A), day 9 (B), and norepinephrine (NE; 1 µM)-treated myocytes (C). Currents were elicited by voltage-clamp steps from a holding potential of −80 mV to −30 to +60 mV.

medium (GIBCO) with the following additions: nonessential amino acids (GIBCO), vitamins (2X; GIBCO); 10 µg/ml insulin (Sigma), 10 µg/ml transferrin (GIBCO); 5% fetal bovine serum, and 100 U/ml penicillin-100 µg/ml streptomycin. Cells were incubated at 37°C in 5% CO₂ in a humidified environment. For the first 7 days in culture, cytosine β-D-arabinofuranoside (10 µM; Sigma) was added to the medium to prevent fibroblast proliferation. Culture medium was changed every 3 days. Culture medium was removed before drug treatment, and the cells were maintained in serum-free medium from the time of drug exposure until Iₒ was measured 24 h later. On the day of electrophysiological recordings, the myocytes were washed three times with sterile PBS and covered with a trypsin-EDTA solution (0.05%, Sigma) until cells were free floating.

Voltage-clamp technique. Current was measured in the whole cell configuration (11) with the use of an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA) interfaced with a personal computer (Dell System 320LX), as described in detail previously (23–25). Data acquisition and analysis were performed using a commercial program (pCLAMP, v. 5.5.1, Axon Instruments). Current records were filtered at 5 kHz and sampled at a frequency of 10 kHz. After data analysis had been performed, the current traces were imported into AcqKnowledge 3.2 (BIOPAC Systems), where they followed. The decay of Iₒ was analyzed using a single exponential fit to a Boltzmann function, Iₒ(max) = 1/1 + exp [(V - m/V)/k], where Vₒ is the membrane potential, m is the membrane potential at which half-inactivation occurs, and k is the slope factor when Vₒ = Vₒ/2. Recovery from inactivation was examined with the use of a double-pulse protocol, in which the time interval between two 300-ms duration pulses to +40 mV from a holding potential of −80 mV was varied between 5 and 750 ms. Cell capacitance was measured by integrating the area beneath the capacitive transient elicited by 10-mV depolarizing steps and dividing that area by the change in voltage.

Study groups. The groups of myocytes studied were as follows: 1) day 0 myocytes, from which recordings were made on the day of isolation; 2) day 9 myocytes, from which recordings were made on day 9 of culture; and 3) drug treatment myocytes, which were exposed to drug treatment for 1 h on day 8 of culture and from which recordings were made on day 9. Drug treatments included NE (1 µM), actinomycin D (1 µg/ml), isoproterenol (ISO; 1 µM), phenylephrine (PE; 1 µM), phorbol 12-myristate 13-acetate (PMA; 0.1 mM), and A-23187 (1 µM).

Data are reported as means ± SE. Statistically significant differences between groups were evaluated initially using
RESULTS

Time-dependent reduction of $I_{to}$. $I_{to}$ density was reduced from $10.4 \pm 0.4 \, \text{pA/pF}$ on day 0 ($n = 29$) to $7.8 \pm 1.6 \, \text{pA/pF}$ on day 5 ($n = 12$) to $3.2 \pm 0.4 \, \text{pA/pF}$ on day 9 ($n = 32$) of culture. Little or no current was recorded from 6 myocytes after 14 days in culture. Given these results, we elected to determine the effects of NE and other interventions on $I_{to}$ after 9 days in culture. At this time, $I_{to}$ density was significantly reduced, but $I_{to}$ could still be measured and current kinetics determined reliably.

Restoration of $I_{to}$ by NE. $I_{to}$ density was significantly reduced in day 9 cells compared with day 0 cells (Figs. 1 and 2). Exposure of myocytes to NE (1 µM) on day 8 of culture had no significant effect on $I_{to}$ within 2 h of exposure (not shown) but increased $I_{to}$ density 24 h after exposure (Figs. 1 and 2). The restoration of $I_{to}$ did not occur in the presence of the transcriptional inhibitor actinomycin D ($I_{to}$ density $= 3.7 \pm 1.3 \, \text{pA/pF}$; $n = 5$).

The kinetics of $I_{to}$ also were altered with time in culture. The time constant of current decay ($\tau_{\text{decay}}$) at +40 mV was significantly increased in day 9 cells compared with day 0 cells (48.6 ± 2.8 vs. 29.3 ± 0.9 ms) (Fig. 3), and the voltage dependence of steady-state inactivation was shifted to more positive potentials ($V_{1/2} = -20.3 \pm 1.9$ vs. $-30.5 \pm 0.8$ mV) (Fig. 3). In addition, the rapid time constant of recovery from inactivation ($\tau_1$) was slightly, but significantly, increased in day 9 cells compared with day 0 cells, and the slow time constant of recovery from inactivation ($\tau_2$) was markedly prolonged (Fig. 4).

Although NE restored $I_{to}$ density, it did not restore $\tau_{\text{decay}}$, which remained prolonged (Fig. 3), or alter the voltage dependence of steady-state inactivation ($V_{1/2} = -19.3 \pm 2.2$ mV) (Fig. 3). The rapid and slow time constants of recovery from inactivation also were not altered by NE exposure (Fig. 4).

To determine whether the induction of $I_{to}$ by NE in the cultured myocytes was mediated by an $\alpha_1$-adrenergic receptor pathway, as in chagasic myocytes (12), myocyte cultures were treated with the $\alpha_1$-adrenergic agonist PE (1 µM) or the protein kinase C activator PMA (0.1 mM). As shown by the normalized current-voltage relationships in Fig. 5, neither PE nor PMA exposure increased $I_{to}$ density. Given these results, we then tested whether the induction of $I_{to}$ by NE was mediated by $\beta$-adrenergic receptors. Exposure to 1 µM ISO restored $I_{to}$ density to day 0 levels (Fig. 6). However, after exposure to ISO, the time course of recovery from inactivation remained slowed (see Fig. 8).

Restoration of $I_{to}$ secondary to activation of $\beta$-adrenergic receptors could be mediated by a number of effects, including elevation of intracellular Ca$^{2+}$. To test whether elevated intracellular Ca$^{2+}$ could restore $I_{to}$, myocyte cultures were exposed to the calcium ionophore A-23187 (1 µM) for 1 h on day 8, and $I_{to}$ was...
measured on day 9. A-23187 increased \( I_{\text{to}} \) density (Fig. 7) but, like Iso, did not restore normal kinetics of recovery from inactivation (Fig. 8).

**DISCUSSION**

\( I_{\text{to}} \) in cultured adult ventricular myocytes. The results of this study indicate that after 9 days in primary culture, peak \( I_{\text{to}} \) density was significantly decreased, whereas \( \tau_{\text{decay}} \) was increased and the time course of recovery from inactivation was prolonged. In addition, \( V_{\text{1/2}} \) was shifted to less negative potentials. These results are similar to those reported previously by Schackow et al. (28), who studied alterations of \( I_{\text{to}} \) in cultured adult feline ventricular myocytes. In those studies, a 50% reduction in \( I_{\text{to}} \) density occurred after 7–9 days in culture, with virtual disappearance of the current within 2 wk. The mechanism for the reduction of \( I_{\text{to}} \) was not identified but apparently did not involve decreased \( I_{\text{to}} \) availability or the expression of a noninactivating \( I_{\text{to}} \). In addition, the reduction of \( I_{\text{to}} \) could not be attributed to the cell culture procedure per se or to the presence of serum in the cell culture medium.

Developmental regulation of \( I_{\text{to}} \). \( I_{\text{to}} \) is known to be developmentally regulated in canine ventricular myocardium (13, 25). Maturation of \( I_{\text{to}} \) is particularly marked between 10 and 20 wk of age (13, 25), a time of parallel maturation of the sympathetic nervous system (32). Consequently, it has been proposed that the developmental changes in \( I_{\text{to}} \) may be regulated by functional sympathetic innervation (13). Previous studies (33, 34) have provided evidence for a trophic effect of sympathetic innervation on the development of L-type \( \text{Ca}^{2+} \) channels (22) and on the maturation of \( \text{Na}^{+} \) current (33, 34). However, there is little direct evidence to support a role for the sympathetic nervous system in the regulation of \( I_{\text{to}} \) development.

Given that the density of \( I_{\text{to}} \) is lower in neonatal ventricular myocytes than in adult myocytes (13, 25), the reduction of \( I_{\text{to}} \) in cultured adult ventricular myocytes may represent a return to a "fetal program" of gene expression (15, 16). If the normal maturation of \( I_{\text{to}} \) is facilitated by, or even dependent on, sympathetic innervation of developing myocytes, it might be expected that the reduction of \( I_{\text{to}} \) in cultured adult ventricular myocytes could be reversed by exposure to sympathetic neurotransmitters. Our observation that the reduction of \( I_{\text{to}} \) in cultured myocytes was reversed by brief exposure to NE and that this effect was prevented by actinomycin D supports this idea.

Sympathetic regulation of \( I_{\text{to}} \) in diseased myocardium. If \( I_{\text{to}} \) gene expression is regulated by the sympathetic nervous system, NE would be expected to restore \( I_{\text{to}} \) in cells in which \( I_{\text{to}} \) has been reduced by a disease process that destroys sympathetic nerves or otherwise interferes with neuronal release of NE. Recently, we have shown that \( I_{\text{to}} \) is reduced during the acute stage of Chagas’ disease in dogs but returns to normal in a more chronic stage of the disease (23). The acute stage of the disease is associated with marked parasitemia (23) and degeneration of sympathetic nerve terminals (18), whereas, in the chronic stage, the parasitemia abates (23) and sympathetic nerve terminals reappear (18). Exposure of acutely infected chagasic myocytes to NE restores \( I_{\text{to}} \) 20–24 h after the initiation of NE exposure, suggesting that the reduction of \( I_{\text{to}} \) during the acute stage of the disease is caused by the loss of a trophic effect of sympathetic innervation. The effects of NE required binding to an \( \alpha_{1} \)-adrenergic receptor and
activation of protein kinase C, with the latter effect most likely involving a pertussis toxin-insensitive G protein and activation of phospholipase C (12).

The reduction of $I_{\text{to}}$ in the hearts of German shepherd dogs with inherited ventricular arrhythmias also may be linked to abnormal sympathetic innervation. $I_{\text{to}}$ is reduced in left ventricular, but not right ventricular, epicardial and Purkinje myocytes isolated from the hearts of affected dogs (9). Sympathetic innervation, as assessed with the use of $[^{123}]$iodoamphetamine (11) and immunohistochemical localization of tyrosine hydroxylase, is reduced in the left ventricle, but not in the right ventricle, of these dogs (5). In an initial attempt to determine whether the association between reduced $I_{\text{to}}$ and denervation was merely coincidental, we tested whether chronic NE exposure restored $I_{\text{to}}$ in left ventricular epicardial myocytes isolated from affected dogs (26). Our results indicate that NE increases $I_{\text{to}}$ density, suggesting that the reduction of $I_{\text{to}}$ in affected myocytes may reflect the lack of a trophic effect of NE on expression of $I_{\text{to}}$.

$I_{\text{to}}$ also is known to be reduced in other forms of cardiac disease, such as subacute myocardial infarction (17), myocardial hypertrophy (31), hypertrophic cardiomyopathy (21), pacing-induced heart failure (14), and X-linked muscular dystrophy (24). It remains to be determined whether the loss of $I_{\text{to}}$ in failing, hypertrophied, or infarcted hearts is related to the abnormalities of sympathetic function that frequently accompany such disease states.

Demonstration that the development of $I_{\text{to}}$ is regulated by sympathetic innervation would require that NE increase the expression of the gene encoding the $I_{\text{to}}$ channel protein. Several candidates for the $I_{\text{to}}$ channel protein exist, including Kv1.4, Kv4.2, and Kv4.3. In rat and ferret myocardium, it appears that Kv4.2 is the $I_{\text{to}}$ channel protein, given that expression of this protein in oocytes produces a current with electrophysiological properties similar to those of native $I_{\text{to}}$ (2) and that messages for both the Kv4.2 (4, 6) and the Kv4.2 protein (1) are abundant in ventricular myocardium. However, recent studies have indicated that Kv4.2 mRNA levels are low in adult canine ventricular myocardium; $I_{\text{to}}$ in this tissue appears to be most similar to Kv4.3 (7). When expressed in oocytes, the Kv1.4 protein also is associated with a transient $K^+$ current, but the recovery kinetics of this current are significantly slower than those of native $I_{\text{to}}$ (27). Further studies are needed in cultured canine myocytes to determine whether NE exposure induces the native $I_{\text{to}}$ alp-subunit but fails to induce a regulatory component of the channel (e.g., a $\beta$-subunit) that is responsible for normal kinetics or whether NE may be inducing the expression of a different transient $K^+$ channel, such as Kv1.4.

Mechanism for the trophic effect of NE on $I_{\text{to}}$. The mechanism for the induction of $I_{\text{to}}$ by NE and isoproterenol is illustrated in Figs. 7 and 8. Activation of protein kinase C, with the latter effect most likely involving a pertussis toxin-insensitive G protein and activation of phospholipase C (12).
end remains to be determined. β-Adrenergic stimulation has not been shown to directly stimulate mitogen-activated protein kinase (MAPK) pathways involved in activation of transcription factors. However, β-adrenergic stimulation results in an elevation of intracellular Ca\(^{2+}\) via the activation of voltage-gated Ca\(^{2+}\) channels. Ca\(^{2+}\) has been shown to be involved in signal transduction pathways such as the MAPK pathways (3, 8) or calcium binding pathways (10, 29, 30), ultimately influencing gene expression through the induction of transcription factors. In this regard, Mori et al. (19) have shown that the induction of Kv1.5 by cAMP derivatives may be mediated by a cAMP response element-cAMP response element binding protein binding site on the 5’ flanking region of the Kv1.5 gene.

Another unresolved issue relates to the observation that the effects of NE on \(I_{\text{to}}\) in chagasic myocytes were mediated by β-adrenergic receptors (12), whereas the effects of NE in the cultured myocytes were mediated by β-adrenergic receptors. In general, the electrophysiological and inotropic effects of β-adrenergic agonists, at least as far as the induction of \(I_{\text{to}}\) is concerned, presently is not clear. It seems possible that α-adrenergic receptors or elements of their associated signaling cascade may be altered by the culture procedure. Consequently, regulation of \(I_{\text{to}}\) in cultured cells may differ in important respects from that in the intact heart.

We thank M. Lisa Lee and Dr. Steven C. Barr for assistance with the cell culture procedures and Dr. Mark S. Roberson for helpful discussions.

Address for reprint requests: Robert F. Gilmour, Jr., Dept. of Physiology, TB 0128 VRT, Cornell Univ, Ithaca, NY 14853-6401.

Received 15 April 1998; accepted in final form 30 July 1998.

REFERENCES


25. Paciorety, L. M., and R. F. Gilmour, Jr. Developmental changes of action potential configuration and \(I_{\text{to}}\) in canine


