STIMULATION OF CARDIAC ALPHA RECEPTORS INCREASES Na/K PUMP CURRENT AND DECREASES g_K VIA A PERTUSSIS TOXIN–SENSITIVE PATHWAY

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ABSTRACT Alpha-adrenergic amines exert concentration-dependent actions on the automaticity of cardiac Purkinje fibers (Posner, P., E. L. Farrar, and C. R. Lambert. 1976. *Am. J. Physiol.* 231:1415–1420; Rosen, M. R., A. J. Hordof, J. P. Ilvento, and P. Danilo, Jr. 1977. *Circ. Res.* 40:390–400; Rosen, M. R., R. M. Weiss, and P. Danilo, Jr. 1984. *J. Pharmacol. Exp. Ther.* 231:1415–1420). At high concentrations they induce a largely beta adrenergic increase in the spontaneous firing rate of adult canine Purkinje fibers, whereas at concentrations $<10^{-6}$ M, their effect is mediated through alpha-adrenergic receptors and is seen predominantly as a decrease in the fibers' spontaneous firing rate. The mechanism for this decrease in spontaneous firing rate remains unexplained. We report here that phenylephrine (10^{-7} M) increases the activity of the Na/K pump and decreases background g_K in Purkinje myocytes. Both effects appear to be alpha-1 adrenergic and, in addition, are abolished on pretreatment with pertussis toxin. These results suggest that like the atrial muscarinic receptor (Pffafinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. 1985. *Nature [Lond.].* 317:536–538; Breitwieser, G. E., and G. Szabo. 1985. *Nature [Lond.].* 317:538–540) the Purkinje fiber alpha-1 receptor is coupled to background g_K via a GTP-regulatory protein. Further, they suggest that the phenylephrine-induced decrease in spontaneous firing rate is due to stimulation of the Na/K pump via a novel coupling of the Na/K pump to a pertussis toxin–sensitive GTP regulatory protein.

INTRODUCTION

Cardiac rate and rhythm are greatly influenced by the sympathetic nervous system, and catecholamines play an important role in the modulation of normal rhythm and the genesis of cardiac arrhythmias (1). Traditionally, catecholamines are believed to affect cardiac rhythm via beta adrenergic receptor stimulation. However there is growing evidence for important alpha-adrenergic effects as well (2–4). Adult canine cardiac Purkinje fibers exhibit a consistent concentration-dependent response to alpha-adrenergic agonists such as phenylephrine (3, 4). At concentrations $\geq 10^{-6}$ M, a largely beta-adrenergic induced increase in automaticity is observed. At phenylephrine concentrations $<10^{-6}$ M, the majority of Purkinje fibers exposed exhibit a decrease in the spontaneous firing rate. This decrease in spontaneous firing rate was found to be mediated by alpha-1 adrenergic receptors (4) and, further, was abolished by pretreatment with pertussis toxin in both neonatal rat ventricle tissue culture and in isolated adult canine Purkinje fibers (5, 6). Because alpha agonists do not alter pacemaker current (7, 8) it is likely that the phenylephrine-induced decrease in spontaneous firing rate is mediated through other membrane currents. We have investigated the ionic mechanism underlying this action of phenylephrine, in isolated canine Purkinje myocytes. We have also investigated the coupling mechanism by which phenylephrine exerts these effects.

METHODS

Acutely dissociated canine Purkinje myocytes (for dissociation procedure see reference 9) were voltage clamped using the whole cell patch clamp technique in the switched configuration (Axoclamp 2; Axon Instruments, Inc., Burlingame, CA). These cells frequently contain little or no pacemaker current. The advantage of using the switched single electrode voltage clamp is that electrode resistance changes do not introduce errors in the measured voltage, because current is not passed while voltage is measured. The data were recorded on an FM tape recorder (model 3964a, 1⅞ ips, 600 Hz bandwidth; Hewlett-Packard Co., Palo Alto, CA). The external solution was a gassed (95%O_2–5%CO_2) Tyrode solution containing (in mM): 140 NaCl, 8 KCl, 1 $CaCl_2$, 12 $NaHCO_3$, 0.4 NaH_2PO_4, 1.6 $MgCl_2$, 10 dextrose, 25 Taurine, 5 β-hydroxybutyric acid and 5 Na-pyruvate. A temperature-controlled experimental chamber maintained a temperature of 35.5 ± 1°C during all experiments (10).

Exposure to Pertussis Toxin

In some experiments designed to investigate the involvement of G proteins in mediating phenylephrine's effects before dissociation, Purkinje fibers were exposed to pertussis toxin. The fibers were stored in tissue culture medium (Medium 199 with Earle's salts; Gibco Laboratories, Grand Island, NY) containing 100 U/ml penicillin and 100 μg/ml streptomycin and 0.5 μg/ml pertussis toxin (List Biologicals, Campbell, CA) for 18–22 h. We employed this concentration of pertussis toxin because

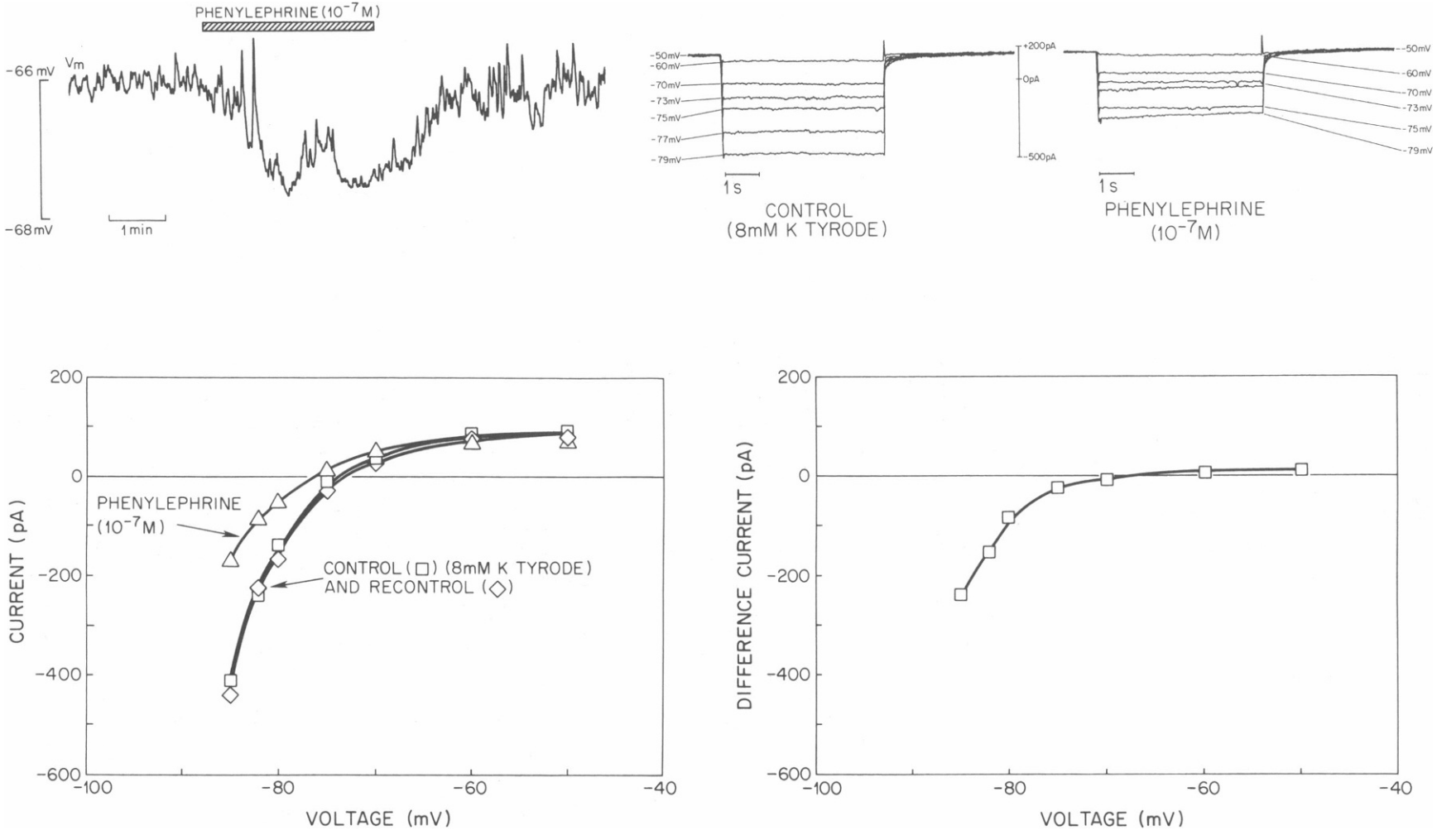

FIGURE 1 Effect of phenylephrine (10^{-7} M) on isolated Purkinje myocytes. (*Upper left*) Effect of phenylephrine on resting membrane potential. Control solution was 8 mM K Tyrode. Bar indicates the duration of phenylephrine exposure. (*Upper right*) Effect of phenylephrine on membrane currents under whole cell voltage clamp conditions. (*Lower left*) Effect of phenylephrine on the "steady-state" membrane I-V relation. Holding potential, −50 mV; pulse duration, 5 s. (*Lower right*) Difference I-V relation, *Control–Phenylephrine*. Similar results on I-V relations were obtained in 12 experiments.

exposure of Purkinje fibers to 0.5 μg/ml pertussis toxin has been demonstrated to abolish the phenylephrine-induced decrease in spontaneous firing rate (5, 6). This effect of pertussis toxin (0.5 μg/ml) is believed to be achieved through maximal ADP ribosylation of a 41-kD protein (presumably the alpha subunit of a G protein), because the pertussis toxin dose response curve for ADP ribosylation correlates with the dose response curve demonstrating abolition of the phenylephrine-induced decrease in the spontaneous firing rate. Lower concentrations of pertussis toxin produced fractional effects (6). We used two types of controls. In one (n = 12) we stored Purkinje fibers for 18–22 h in the tissue culture medium, but without pertussis toxin. These fibers were then dissociated according to the usual protocol, and the dissociated cells' response to phenylephrine was examined. Results indicate that in the absence of pertussis toxin this storage period had no effect on the myocytes' sensitivity to phenylephrine. In these experiments the control Purkinje fibers and the pertussis toxin–pretreated Purkinje fibers were not obtained from the same animal. Therefore, we performed a second type of control (n = 2), where we compared cells dissociated from Purkinje fibers from the same animal. One batch was dissociated immediately while the second was stored with pertussis toxin (0.5 μg/ml). This type of control was performed to rule out the possibility that pertussis toxin–treated cells were phenylephrine-insensitive before being treated with pertussis toxin. Results from such experiments indicated that cells from the same animal exhibited normal sensitivity to phenylephrine before being treated with pertussis toxin.

The patch pipette contained (in mM): 117 KCl, 23 KOH, 10 NaCl, 2 $MgCl_2$, 10 dextrose, 11 EGTA, 10 Hepes, 1 $CaCl_2$, and 100 μM GTP; pH was adjusted to 7.4. The pipette resistance before sealing was 1–3 Mohms. ATP was not routinely included in the pipette solutions. Four control experiments were performed in which ATP (2 mM) and creatinine phosphate (3 mM) were added to the pipette solution. They demonstrated identical effects of phenylephrine, suggesting that the presence of ATP is not critical for phenylephrine's effects. We believe that this absence of a pipette-ATP requirement for Na/K pump stimulation points to a slow exchange between pipette constituents and the large cells we studied (average dimensions: length, 180 μm; radius, 15 μm). In a separate set of experiments (11) when pipette [K^+] was reduced from 140 to 25 mM it took >30 min for the exchange to be nearly complete. Because the diffusion coefficient for ATP is lower than that for K^+, an even slower time course for the exchange of cellular ATP is expected. Furthermore, calculations modeling this diffusion process using the equations of Oliva et al. (12) suggest that exchange of ATP in our cell with our pipettes should take many tens of minutes. The slow loss of ATP is also supported by the ready reversibility of phenylephrine's effects and the constant slope conductance in control solutions throughout the duration of the experiment. If ATP levels dropped below 1 mM the ATP-sensitive K^+ conductance should be unveiled (13) and the membrane slope conductance would be expected to increase. No such change was observed.

Measurement of Zero-Current Potential

One of the parameters we used to examine the effects of phenylephrine on Purkinje myocytes was the shift in the zero-current potential (under voltage clamp conditions) induced by phenylephrine. To obtain the zero-current potential from the recorded total cell I-V relation, the I-V relation was fit using least squares with a fourth or fifth order polynomial. The intersection of this fit with the voltage axis was taken to be the zero-current potential.

RESULTS

Fig. 1 (*upper left*) illustrates the effect of 10^{-7} M phenylephrine on the resting potential (V_m) of a Purkinje myocyte. As seen, the myocyte reversibly hyperpolarizes in the presence of the alpha agonist. To investigate the mechanism underlying this hyperpolarization, we voltage clamped Purkinje myocytes and recorded the currents elicited in response to voltage steps (5-s duration) in control solution and then in the presence of phenylephrine (10^{-7} M). Such an experiment is illustrated in the upper right panel of Fig. 1. The holding potential was −50 mV, and voltage steps in the negative direction were applied. The current recorded in response to the voltage steps was found to be less inward in phenylephrine. Fig. 1 (*lower left*) illustrates the effect of phenylephrine on the cell I-V relation, negative to −50 mV. The inward current was reduced in the presence of the drug, and the zero-current potential shifted in the negative direction (average shift, −2.6 ± 0.6 mV [SEM, n = 12]). The difference I-V relation, which represents the component of the total membrane I-V relation affected by phenylephrine is shown in the lower right panel of Fig. 1. This current is inwardly rectifying like many K^+ currents, but does not reverse at the predicted E_K (K_o = 8 mM; pipette K = 140 mM; E_K = 76 mV).

Although phenylephrine predominantly stimulates alpha-adrenergic receptors, it may have beta effects as well. To investigate this possibility the experiments shown in Fig. 2 were performed. In the presence of 2 × 10^{-7} M propranalol, a beta antagonist, the zero current potential is shifted in the negative direction (similar to Fig. 1, *lower*

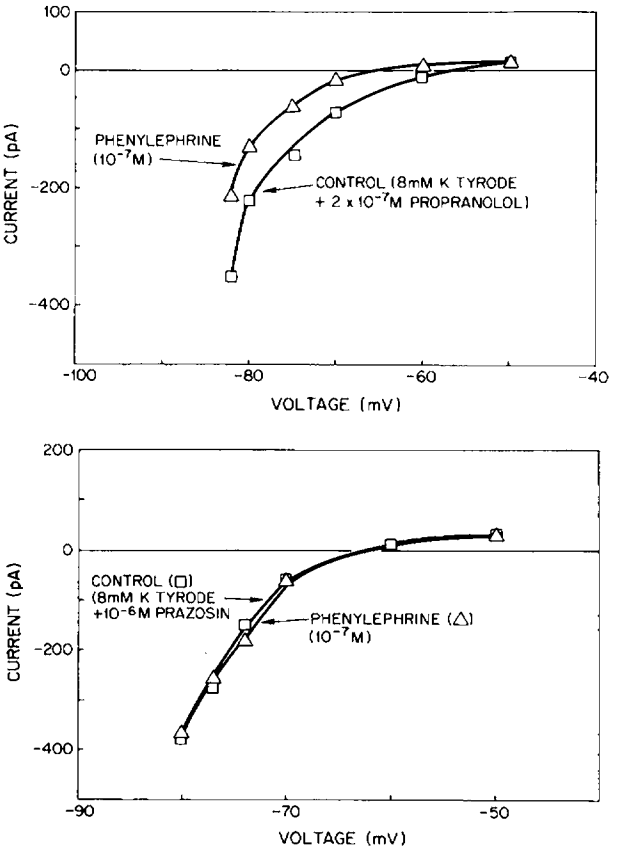

FIGURE 2 Effect of phenylephrine on the membrane I-V relation, in the presence of propranolol (*top*) and prazosin (*bottom*). Holding potential, −50 mV; pulse duration, 5 s.

left) and the inward current reduced (Fig. 2, *top*, $n = 4$). However, in the presence of 10^{-6} M prazosin (an alpha-1 antagonist), phenylephrine elicits no response (Fig. 2, *bottom*, $n = 3$). These results suggest that phenylephrine (10^{-7} M) acts primarily through alpha-1 receptors.

The decreased slope of the inward rectifying region of the membrane I-V relation in phenylephrine (Fig. 1, *lower left*) suggests that phenylephrine decreases background g_K. (A preliminary report has suggested that the alpha-agonist methoxamine decreases membrane potassium permeability in Purkinje fibers [14].) Consistent with this suggestion, measurements of the slope conductance of the cell in control solution and in phenylephrine show a decreased slope conductance in phenylephrine (Fig. 3, *top*). On its own, decreasing background g_K would tend to depolarize the cell; in fact the opposite is observed (Fig. 1, *upper left*). (This effect, i.e., hyperpolarization induced by phenylephrine [10^{-7} M] has been previously shown to occur in intact Purkinje fibers [3].) Therefore, phenylephrine, in addition to decreasing background g_K, must have a second effect on Purkinje cells. To unveil this second effect we employed 1 mM Ba^{2+} to block background g_K. In the presence of Ba^{2+} phenylephrine shifted the I-V relation outward, in an approximately voltage-independent manner (Fig. 3, *lower left*). The outward shift was 41 ± 19 pA (SEM, $n = 4$) at V = −70 mV. We next examined the actions of phenylephrine in the presence of both 1 mM Ba^{2+} and 10^{-4} M dihydroouabain (DHO) ($K_D = 4 \times 10^{-6}$ M) (to block background g_K and the Na/K exchange pump, respectively). Under these conditions, phenylephrine had no effect on the membrane I-V relation (Fig. 3, *lower right*, $n = 4$). These results indicate that phenylephrine, in addition to decreasing background g_K, also increases the outwardly directed Na/K pump current.

Because phenylephrine hyperpolarizes the cell despite reducing K^+ conductance, it seems likely that this hyperpolarization is a consequence of phenylephrine's stimulation of Na/K pump current. If the Na/K pump is blocked (using dihydroouabain), phenylephrine should, under voltage clamp conditions, shift the myocyte's zero-current potential in the positive direction. (This corresponds to a depolarization in an unclamped cell.) Experiments per-

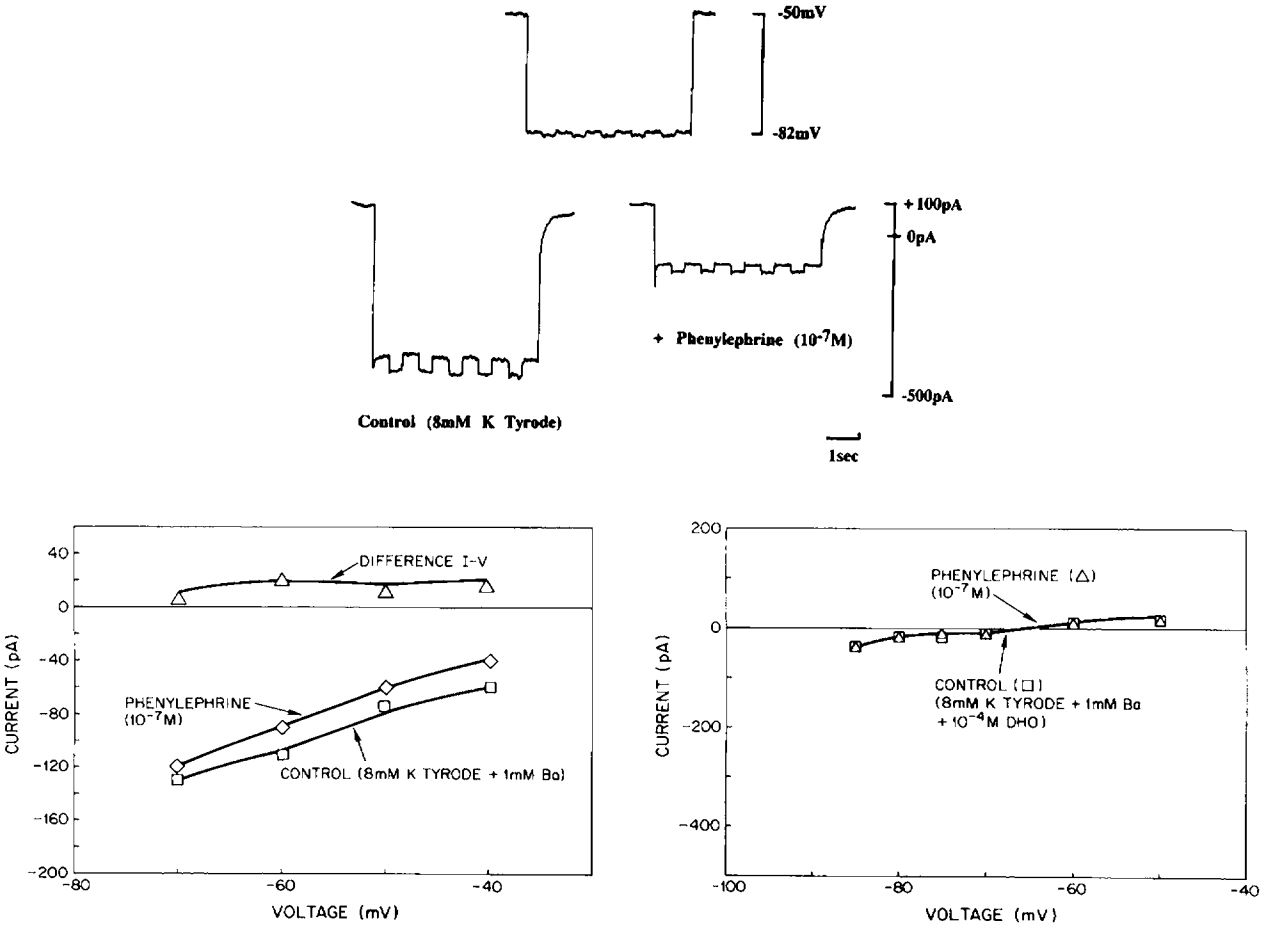

FIGURE 3 (*Top*) Phenylephrine's effects on slope conductance. Holding potential, −50 mV. Pulse duration, 5 s. Superimposed voltage steps, 1 mV in amplitude and 400 ms in duration ($n = 4$). (*Lower left*) Phenylephrine's effects on the steady-state membrane I-V relation in the presence of 1 mM Ba^{2+}. Difference I-V relation is *Phenylephrine–control*. (*Lower right*) The effect of phenylephrine in the presence of Ba^{2+} and DHO, which should block the background K^+ current and the Na/K exchange pump current. Holding potential, −50 mV; pulse duration, 5 s.

formed in the presence of DHO (10^{-4} M) indicate that there is indeed an increase in inward current at the original zero-current potential which should result in a depolarization in an unclamped cell (Fig. 4, *top*). Furthermore, if the increase in Na/K pump current induced by phenylephrine, which would have occurred had the pump not been blocked by DHO, 41pA, is compensated for, the resulting membrane I-V relation in phenylephrine resembles that in Fig. 1, lower left panel (Fig. 4, *bottom*). Therefore, the effect of phenylephrine on the Na/K pump, together with its effect on background g_K, can account for its effect on the total membrane I-V relation negative to −50 mV.

Studies in atrial cells have implicated GTP and an N_i-like GTP-regulatory protein in mediating the effects of muscarinic agonists on background g_K. Pertussis toxin, by specifically ADP-ribosylating N_i-like GTP-regulatory proteins, blocks signalling systems involving these proteins (15). Previously, pertussis toxin has been shown to eliminate the negative chronotropic effect of phenylephrine in neonatal rat ventricular myocytes in tissue culture and in intact canine Purkinje fibers (5, 6). To determine if GTP-regulatory proteins are involved in mediating phenylephrine's effects on background g_K and on the Na/K pump, we pretreated Purkinje fibers (18–22 h) with 0.5 μg/ml pertussis toxin (for procedure and controls performed see Methods) before dissociation. As Fig. 5 (left upper and lower panels and upper right panel) demonstrates, such cells demonstrated no response to phenylephrine, indicating that an N_i-like GTP-regulatory protein is likely to be involved in the mediation of phenylephrine's effects.

A second method to test the involvement of GTP and GTP-regulatory proteins in the mediation of phenylephrine's effects is to substitute for the GTP in the pipette with a nonhydrolyzable analogue such as GppNHp. Under these conditions, uncoupling of the alpha receptors from the Na/K pump and the background K^+ conductance occurs, so that once the system has been turned on by the agonist it cannot be turned off, even after removal of the agonist from the bathing solution. In such experiments using GppNHp, 68 ± 9% (SEM, $n = 5$) of the effect at −75 mV remained after phenylephrine was removed from the bathing solution (Fig. 5, *lower right*), also supporting the involvement of GTP in the mediation of phenylephrine's effects.

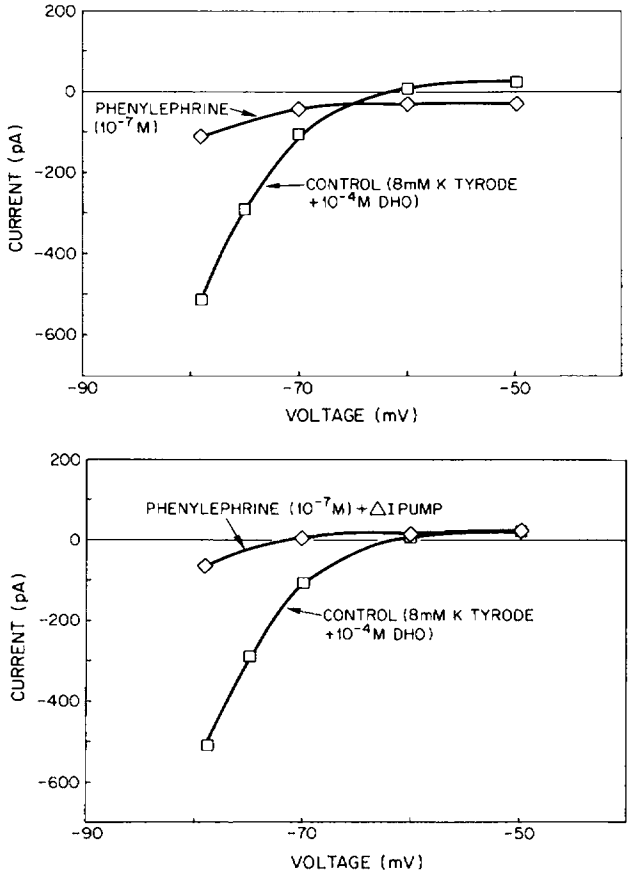

FIGURE 4 (*Top*) Effect of phenylephrine in the presence of DHO ($n = 4$). (*Bottom*) (□) Control I-V relation (from A); (◇) phenylephrine I-V relation (from A) + 41 pA (average phenylephrine induced increase in I_{pump} [see Results]).

DISCUSSION

Our results suggest that phenylephrine reduces background K^+ conductance while increasing the current due to electrogenic Na/K exchange. To reach this conclusion we employed 1 mM Ba^{2+} and 10^{-4} M dihydroouabain as selective blockers of the K^+ conductance and the Na/K pump current, respectively. Ba^{2+} has long been known to block inwardly rectifying K^+ currents in a variety of tissues (16–18). DiFrancesco (1981) (18) first employed it in Purkinje fibers as a tool to separate the inwardly rectifying K^+ current, I_{K1}, from other diastolic membrane currents. We have previously shown in both canine Purkinje fibers (19) and in isolated canine Purkinje myocytes (20) that 1 mM Ba^+ is an effective concentration to entirely block the inwardly rectifying K^+ current I_{K1}. Similarly the glycosides have long been used as selective blockers of the Na/K exchange pump. Isenberg and Trautwein (1974) (21) first demonstrated the virtues of the rapidly acting dihydroouabain as an experimental tool to separate the Na/K pump current from other membrane currents in Purkinje fibers. Cohen et al. (9) have characterized the kinetics of DHO inhibition of Na/K pump current in isolated canine Purkinje myocytes. The K_D was 3.7×10^{-6} M. The concentration we employed (10^{-4} M) should block >95% of the pump current.

Catecholamine stimulation of the Na/K pump in cardiac muscle has been previously reported (22, 23) but is widely believed to occur via a beta-adrenergic action. We find that the Na/K pump in Purkinje myocytes is also stimulated by phenylephrine, acting through alpha-1 receptors. Further, the Na/K pump interacts with the alpha-1 receptor through GTP and GTP-regulatory proteins. Phenylephrine's effect on the Na/K pump could be

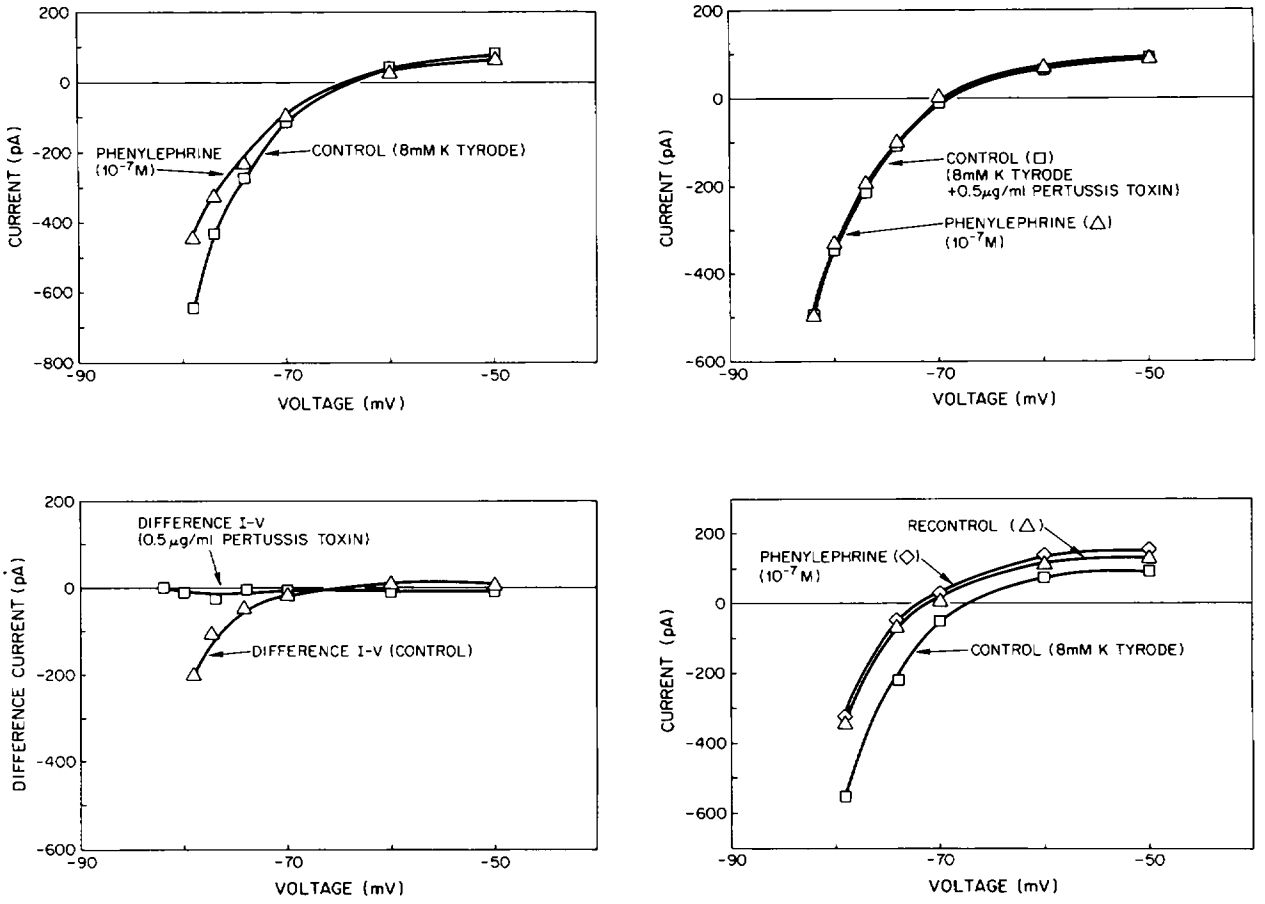

FIGURE 5 Effect of phenylephrine on the I-V relations of cells from fibers pretreated with 0.5 μg/ml pertussis toxin (18–22 h) before dissociation (see Methods for procedure). (*Upper left*) Control experiment (cells examined on the first day; see Methods for the two types of control experiments performed). (*Upper right*) Cell from fibers from the same animal as *A*, pretreated with pertussis toxin (18–22 h) ($n = 7$). (*Lower left*). Difference I-V relations from *Control* experiment and from pertussis toxin–treated cells. Holding potential, −50 mV; pulse duration, 5 s. (*Lower right*) Effect of phenylephrine when pipette GTP is substituted with 100 μM GppNHp. In five such experiments, 68 ± 9% (SEM) of the effect at −75 mV remained after phenylephrine was removed from the bathing solution.

either direct or indirect. Evidence suggesting that the stimulation of the Na/K pump is not mediated indirectly by a rise in $[Na^+]_i$ comes from preliminary experiments by Zaza et al. (24) and Wang et al. (25), which demonstrate that intracellular $[Na^+]$ in Purkinje fibers falls when the fibers are exposed to phenylephrine (24) or norephinephrine (25). Moreover, in the former study, the effect of phenylephrine was blocked by prazosin but not by propranolol (preliminary data, A. Zaza). This suggests that the action of phenylephrine is either direct or if it is indirect, it occurs not through a rise in $[Na^+]_i$, but through some alternative mechanism.

Phenylephrine may act directly on the background K^+ channels or its effect may be mediated by intracellular messengers such as inositol triphosphate or diacylglycerol, both breakdown products of PI. (The effects of muscarinic agonists on K channels in atrial myocytes seem to be direct [26].) Although we are not certain if this decreased K^+ conductance is through the cardiac I_{KI} channels, preliminary results suggest that this may be the case.

The typical capacitance of our cells is 2.83×10^{-4} μF (9) and the typical conductance 47 μS/μF (27). This yields a typical cell input resistance of 76 Mohms. Given this value of input resistance, a pump current increase of 41 pA (as we see) would be expected to hyperpolarize the cell by ~3 mV. However, a decrease in background g_K tends to depolarize the cell, therefore the actual phenylephrine-induced hyperpolarization is expected to be somewhat less than 3 mV. As stated in the Results, we observe, under voltage clamp, a negative shift in the zero-current potential of 2.6 mV.

Our results explain some of the alpha-adrenergic effects of phenylephrine; at low agonist concentrations the stimulation of the Na/K pump predominates, resulting in membrane hyperpolarization and decreasing automaticity. The increased automaticity of Purkinje fibers in response to the higher concentrations of phenylephrine may be a result of beta-adrenergic stimulation of the pacemaker current (28). In Purkinje fibers, propranolol largely inhibits the increase in spontaneous firing rate at these higher phenylephrine concentrations (4). We are still uncertain of the cause of the increase in spontaneous firing rate seen in

a subset of fibers at low alpha-agonist concentrations (3). This may be the result of a more dominant effect of the alpha-agonist on background g_K, thereby exceeding the Na/K pump stimulation at these concentrations.

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