Effects of Forskolin on Intracellular Sodium Activity in Resting and Stimulated Cardiac Purkinje Fibers from Sheep

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R. K. ZALUPS AND S.-S. SHEU. Effects of Forskolin on Intracellular Sodium Activity in Resting and Stimulated Cardiac Purkinje Fibers from Sheep. Journal of Molecular and Cellular Cardiology (1987) 19, 887–896. In the present investigation, the effects of forskolin on intracellular sodium activity were studied in quiescent and electrically stimulated cardiac Purkinje fibers from sheep using Na+-sensitive microelectrodes. Also assessed, were the effects of this promoter of cytosolic cAMP production on resting membrane potential, action potential and twitch tension. In the quiescent fibers, forskolin (12 μM) caused intracellular sodium activity to decrease in the face of cellular depolarization. This cellular depolarization was occasionally accompanied by spontaneous firing of action potentials. In the stimulated fibers, forskolin (10 μM) also caused intracellular sodium activity to decrease. Moreover, it caused a marked acceleration of phase 4 pacemaker depolarization, an elevation of the plateau of the action potential and an increase in twitch tension. When the Na+ pump was inhibited by either strophanthidin (1 μM) or by 0 mM extracellular K+, forskolin had no effect on intracellular sodium activity. In summary, the results of the present study indicate that forskolin, presumably by increasing intracellular cAMP, causes the following to occur in cardiac Purkinje fibers from sheep: (a) a decrease in intracellular sodium activity when the Na+ pump is functioning normally; (b) a promotion of membrane depolarization in quiescent fibers; (c) an increase in the steepness of the pacemaker potential in electrically stimulated fibers, and (d) an increase in the force of contraction. Therefore, forskolin will be a useful tool for investigating the role of cAMP in physiological function of cardiac cells.

KEY WORDS: Forskolin; Catecholamines; Cyclic AMP; Intracellular sodium activity; Cardiac Purkinje fibers

Introduction

Catecholamines are known to have profound inotropic and chronotropic effects on cardiac muscle. Experimental findings indicate that a number of effects of catecholamines may be brought about by the actions of cytosolic cAMP [2, 25, 38, 41], which increases via a receptor-mediated adenylate cyclase system. An interesting effect of catecholamines, that has been recently observed, is hyperpolarization of maximum diastolic potential associated with a concomitant decrease of intracellular sodium activity (aNa) in canine cardiac Purkinje fibers [15, 44]. A decrease in aNa by isoproterenol has also been observed in isolated cardiac myocytes of rabbit [6]. Based on the findings of two groups of investigators [6, 15], it appears that the decrease in aNa may be predominantly due to stimulation of the Na+ pump. In addition, recent findings indicate that analogues of cyclic AMP also cause aNa to decrease in canine cardiac Purkinje fibers [20]. This implies the effect of catecholamines on aNa may be directly linked to cAMP. In quiescent sheep cardiac Purkinje fibers, however, some investigators have demonstrated that catecholamines cause depolarization of resting membrane potential in association with an increase in aNa [10] or no change in aNa [39]. These investigators suggested that catecholamines induce depolarization of resting membrane potential by activating a pacemaker current, which is carried by the influx of Na+. They excluded the possibility that catecholamines stimulated the Na+ pump in their experiments. Forskolin, which is a diterpene extracted from the root of the Indian plant Coleus forskohlii, like catecholamines, significantly stimulates the

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activity of adenylate cyclase, which in turn increases the production of cytosolic cAMP [16, 29]. What is unique about this particular compound is that it stimulates adenylate cyclase activity at sites beyond the receptor, such as at the catalytic subunit of adenylate cyclase [21, 28] and other regulatory sites for the catalytic subunit [7, 11, 17, 37].

The aim of the present study is to determine whether forskolin causes $a_{\text{Na}}$ to change and, if so, whether this change may be related to the activity of the Na$^+$ pump. In addition, the effects of forskolin on the electrical and mechanical properties of cardiac Purkinje fibers were also examined.

A preliminary report of this work has been presented [31].

**Materials and Methods**

Free-running cardiac Purkinje fibers from sheep were used in the present study. The hearts were obtained from a nearby slaughterhouse and transported to the laboratory in a cooled, oxygenated saline solution, which contained (in mM) 140 NaCl; 5 KCl; 2 CaCl$_2$; 1 MgCl$_2$; 5.5 glucose and was buffered with 5 mM HEPES ([(4-(2-hydroxyethyl)-1-piperazinio)ethanesulfonic acid]). The solution was titrated to pH 7.35–7.40 using 1 N NaOH. The preparations were pinned down onto an elastomer base of a superfusing chamber and bathed with the above mentioned saline solution at 37°C. During experiments all superfusing solutions were continuously gassed with 100% O$_2$.

Forskolin (Calbiochem-Behring, La Jolla, CA, USA) and strophanthidin (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in 100% ethanol to give a stock concentration of 20 to 24 mM. For experiments, the saline solution was used to dilute forskolin to 10 to 12 μM and strophanthidin to 1 μM. The final concentration of ethanol in the superfusing solution never exceeded 0.05%. This concentration of ethanol had no effect on membrane potential and $a_{\text{Na}}$. Two different types of experiments were carried out. One in which the Purkinje fibers were quiescent and the other in which the fibers were stimulated electrically at 1 Hz. In those experiments where fibers were stimulated, one end of each fiber was attached to a Model 405 Cambridge tension transducer (Cambridge Technology, Inc., Cambridge, MA, USA) in order to obtain measurements of twitch tension.

Membrane potential was measured using conventional microelectrodes and a WPI Model KS-700 electrometer (WP Instruments, Inc., New Haven, CT). Na$^+$-sensitive microelectrodes were used to measure $a_{\text{Na}}$. The microelectrodes were made from thin-walled borosilicate tubing (WPI-TW 150 F4; Instruments Inc., New Haven, CT, USA) using the previously published protocol [30, 36]. The Na$^+$-sensitive microelectrodes were made a day or two prior to their use. After each experiment the microelectrode tips were calibrated using solutions made from the following mixtures of NaCl and KCl (in mM): 1 NaCl : 149 KCl; 3 NaCl : 147 KCl; 10 NaCl : 140 KCl; 30 NaCl : 120 KCl; 100 NaCl : 50 KCl. The activity coefficient for Na$^+$ at each solution was assumed to be 0.76. For each microelectrode a calibration curve was constructed by plotting on semi-logarithmic graph paper, the Na$^+$ activity (in mM) of each respective calibrating solution against each corresponding potential (in mV) as measured with a Keithley Model 614 electrometer (Keithley Instruments Inc., Cleveland, OH, USA). The actual determination of $a_{\text{Na}}$ was accomplished by reading from the calibration curve the respective Na$^+$ activity that corresponded to the voltage generated by the Na$^+$ electrochemical gradient. This potential was determined by electronically subtracting the voltage measured with the conventional microelectrode from the voltage measured with the Na$^+$-sensitive microelectrode. In the electrically stimulated fibers, we have removed the artifactual variations of measured $a_{\text{Na}}$ by filtering the intracellular Na$^+$ signal with an eight-pole low-pass Bessel filter (Frequency Devices) with a cut-off frequency between 1 and 0.2 Hz [32].

For obtaining a permanent record of each experiment a Gould Model 2600S chart recorder was used (Gould Inc., Cleveland, OH, USA).

Statistical differences between means, for membrane potential and $a_{\text{Na}}$ before and after exposure to forskolin, were evaluated using the paired Student’s t-test (two-tailed). Differences between means were regarded sta-
Forskolin (1.2 x 10^{-5} M)

10 min

\[ a_{\text{Na}} \text{ (mM)} \]

8.8

\[ \text{Membrane potential (mV)} \]

-75

FIGURE 1. (a) Effects of forskolin (12 \( \mu \text{M} \)) on intracellular sodium activity \( (a_{\text{Na}}) \) and membrane potential in a quiescent cardiac Purkinje fiber from sheep. During the exposure to forskolin, \( a_{\text{Na}} \) decreased from 10.2 to 8.9 mM and membrane potential changed from -74 to -67 mV; (b) Induction of spontaneous firing of a quiescent cardiac Purkinje fiber from sheep following the exposure to forskolin (12 \( \mu \text{M} \)). Forskolin caused a marked depolarization (-71 mV to -60 mV). After removing forskolin from the superfusing solution, while the fiber was still depolarized, spontaneous oscillations began initially and were followed by spontaneous action potentials.

\[ \text{statistically significant when } P \text{ was less than or equal to 0.05.} \]

\section*{Results}

In a total of 11 quiescent fibers, 5 to 10 mins of exposure to forskolin (12 \( \mu \text{M} \)) caused a decrease in \( a_{\text{Na}} \) [Fig. 1(a)], with an average change of \( 2.7 \pm 0.4 \text{ mM (mean } \pm \text{s.e.m., } P < 0.05) \). The \( a_{\text{Na}} \) fell from a resting value of 8.2 \( \pm \) 0.5 mM to a value of 5.6 \( \pm \) 0.6 mM. A surprising finding was that forskolin also caused a marked cellular depolarization. The average change in membrane potential was 11.2 \( \pm \) 0.7 mV \( (P < 0.05) \), from a resting value of -77 \( \pm \) 1.3 mV to a value of -65 \( \pm \) 1.7 mV. Forskolin also induced spontaneous firing in four fibers during depolarization [Fig. 1(b)]. In all four fibers, the onset of spontaneous activity occurred during the washout of forskolin. The effects of forskolin were completely reversible after the removal of the drug. However, the rate of the recovery in resting potential and \( a_{\text{Na}} \) during the washout of forskolin was slower than the rate of the action during the exposure to forskolin.

In the electrically stimulated fibers, 1 to 3 mins of exposure to forskolin (10 \( \mu \text{M} \)) also caused a decrease in \( a_{\text{Na}} \) [Fig. 2(a)]. The average change was 1.1 \( \pm \) 0.2 mM \( (P < 0.05) \) in four fibers, from a control level of 8.4 \( \pm \) 0.5 mM to 7.3 \( \pm \) 0.4 mM. Coincidently, the control \( a_{\text{Na}} \) in stimulated fibers was not different from that in quiescent fibers, perhaps as a result of variation in \( a_{\text{Na}} \) between the two sets of fibers. It has been shown that, in a given cardiac Purkinje fiber from sheep, electrical stimulation at 1 Hz caused \( a_{\text{Na}} \) to increase by 1.4 mM [3]. Samples of action potential indicated by arrows at time C and F in Figure 2(a) are shown in Figure 2(b). Several effects of forskolin were seen on the action potential. First, there was a small decrease in the maximal diastolic potential. Second, there was a pronounced increase in the steepness of the pacemaker depolarization. Third, once this depolarization reached threshold, spontaneous activity occurred. Fourth, the plateau phase of the action potential was increased. The elevation of the

\section*{Discussion}

The results of this study demonstrate that forskolin is a potent activator of intracellular sodium activity and membrane depolarization in cardiac Purkinje fibers. The decrease in \( a_{\text{Na}} \) and the marked cellular depolarization observed during forskolin exposure suggest that forskolin activates a sodium channel, possibly the voltage-gated sodium channel. Moreover, the spontaneous firing induced by forskolin indicates that forskolin may also activate a pacemaker channel, possibly the sodium-potassium ATPase channel. The reversal of the effects of forskolin after washout suggests that forskolin acts as a transient activator of sodium channels, which is consistent with the known pharmacology of forskolin. The results of this study provide new insights into the mechanism of action of forskolin and may have implications for the treatment of cardiac arrhythmias.
plateau phase of the action potential by forskolin is also indicated by the trace of membrane potential in Figure 2(a). In this figure, the peaks of action potential were less than 0 mV, which was due to the filtering of the amplifier in the Gould chart recorder (at a cut-off frequency of 15 Hz). Therefore, the peak of action potential in this figure represented the voltage range around the plateau of action potential. In Figure 2(b), the amplifier of the recorder was not filtered, however, the amplitude of action potential was still partially distorted owing to the intrinsic frequency response of the amplifier (around 1 KHz). In addition to the effects on electrical activities, forskolin also caused an increase in twitch tension. As with the quiescent fibers, all the effects of forskolin were completely reversible.

In order to determine the effects of forskolin on \( \Delta_{Na} \) during the inhibition of the Na\(^{+} \) pump, solutions containing either 1 \( \mu M \) strophanthidin or 0 mM K\(^{+} \) were used. At a concentration of 1 \( \mu M \), strophanthidin caused \( \Delta_{Na} \) to increase from 7.8 \( \pm \) 0.6 mM to 25.3 \( \pm \) 2.7 mM (\( P < 0.05 \)) within 20 to 30 min in seven fibers. During the period of inhibition of the Na\(^{+} \) pump, 12 \( \mu M \) forskolin had no effect on the rate of increase of \( \Delta_{Na} \) [Fig. 3(a)]. Forskolin did, however, cause a cellular depolarization
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FIGURE 3. Effect of forskolin (12 μM) on intracellular sodium activity (aNa) and membrane potential of a resting cardiac Purkinje fiber from sheep exposed to strophanthidin (1 μM). (a) Strophanthidin, which blocks the sodium pump, caused aNa to increase gradually. Forskolin was introduced into the superfusing solution while aNa was still increasing. Forskolin did not cause any change in the time-course of the strophanthidin-induced increase in aNa. Forskolin, however, caused a marked depolarization (10 mV). In addition, forskolin induced oscillations in membrane potential, which can be seen in the trace depicting aNa. (b) Forskolin was introduced at the time that aNa was near the steady state level after exposure to strophanthidin (1 μM). Note that forskolin caused a smaller depolarization in membrane potential (2 mV).

while the Na⁺ pump was being inhibited by strophanthidin. On average, membrane potential depolarized by 4.4 ± 0.8 mV (P < 0.05) in seven fibers. Although the time course of the depolarization and subsequent recovery during washout was quite similar to that seen with forskolin alone, the magnitude of depolarization was smaller (4.4 mV vs 11.2 mV). In Figure 3(b), forskolin was added near the new steady state of aNa, after which a very small depolarization (2 mV) occurred with no influence on the rate of aNa increase. Prolonged exposure to strophanthidin caused most of the fibers to develop small oscillations at the level near the resting membrane potential (0 to 3 mV). Forskolin caused these oscillations to increase in size, as is clearly demonstrated by the tracing of aNa in Figures 3(a) and 3(b). None of the fibers, however, initiated spontaneous action potentials.

When K⁺ was removed from the superfusing solution, a pronounced depolarization (of 30 to 40 mV) occurred with a subsequent attainment of a second level of resting membrane potential. In two out of four fibers the membrane potential was oscillating after the depolarization. Following the application of forskolin (12 μM) these oscillations in membrane potential increased in magnitude [Fig. 4(a)]. In the other two fibers, the second level of resting membrane potential was stable, that made it possible to record aNa. Inhibition of the Na⁺ pump by removing extracellular K⁺ caused aNa to increase to a new steady state (20.5 ± 0.5 mM) within 20 to 30 mins. Forskolin (12 μM) had no effect on the rate of increase of aNa while the Na⁺ pump
FIGURE 4. (a) Effects of forskolin (12 μM) on oscillations in membrane potential induced by removing all K⁺ from the extracellular medium. Removal of K⁺ from the superfusing solution caused a marked depolarization and oscillations in membrane potential. Introduction of forskolin to the superfusing solution caused the amplitude of these oscillations to increase considerably; (b) Effects of 12 μM forskolin on intracellular sodium activity \( [\alpha_{\text{Na}}] \) and membrane potential of a sheep cardiac Purkinje fiber following the removal of extracellular K⁺. Removal of K⁺ from the superfusing solution caused a large depolarization (from -82 to -44 mV). In this case membrane potential remained stable following depolarization. Removing potassium from the superfusing solution caused \( [\alpha_{\text{Na}}] \) to increase (from 7.8 to 22 mM). Forskolin, in the absence of extracellular potassium, had no effect on either \( [\alpha_{\text{Na}}] \) or membrane potential.

was being inhibited by 0 mM K⁺ [Fig. 4(b)]. This is consistent with the findings from the experiments in which the Na⁺ pump was inhibited by strophanthidin. Surprisingly, forskolin had no effect on membrane potential in these two fibers under the above stated conditions.

**Discussion**

In the present study, the diterpene forskolin caused: (1) a decrease in \( [\alpha_{\text{Na}}] \) only when the Na⁺ pump was functioning normally, (2) a depolarization of the membrane potential in quiescent fibers, (3) an increase in the steepness of phase 4 pacemaker potential in stimulated fibers, and (4) an increase in the force of contraction.

It has been demonstrated that catecholamines caused \( [\alpha_{\text{Na}}] \) to decrease in canine cardiac Purkinje fibers [15, 44] and in isolated cardiac myocytes of the rabbit [6]. This decrease in \( [\alpha_{\text{Na}}] \) can be prevented when the Na⁺ pump is inhibited partially by strophanthidin (0.25 μM) [15] or by a K⁺-free solution [20]. Similarly, the decrease in \( [\alpha_{\text{Na}}] \) can be reduced in 1.5 mM [K⁺]o [6]. These findings have led investigators to conclude that catecholamines directly stimulate the Na⁺ pump in cardiac muscle cells [6, 15, 20] as similar to the K⁺ efflux studies in heart [43] and in other excitable cells [22]. Both forskolin and catecholamines stimulate the activity of adenylate cyclase. Forskolin, however, accomplishes this by acting at a level beyond the beta-receptor [7, 11, 17, 21, 28, 37]. Since
CAMP acts as a second messenger in many cellular processes, it is not unreasonable to postulate that CAMP is the mediator of the effects of forskolin and catecholamines on $a_{Na}$ in cardiac muscle. Somewhat more direct evidence supporting this hypothesis comes from a very recent study [20], which demonstrates that analogues of CAMP cause $a_{Na}$ to decrease in canine cardiac Purkinje fibers. As will be discussed later, although the present data show that forskolin reduces $a_{Na}$ only when the Na$^+$ pump is functioning normally, nevertheless, this reduction in $a_{Na}$ is not necessarily due to a direct stimulation of Na$^+$ pump by forskolin.

Another prominent effect of forskolin that was detected in the present study was the induction of a marked depolarization in the resting potential of the quiescent fibers and an augmentation of pacemaking activity in the electrically stimulated fibers. Epinephrine and isoproterenol have also been shown to cause a depolarization in the resting potential of quiescent cardiac Purkinje fibers from sheep [19, 39]. Other investigations, however, have shown that β-adrenergic agonists cause a hyperpolarization in the maximum diastolic potential of canine Purkinje fibers [15, 44]. This hyperpolarization appears to be due to an increase in conductance of potassium [9] or the stimulation of the electrogenic Na$^+$ pump [6, 15]. The reason for the disparity in results between the aforementioned studies is not obvious. One possibility is that catecholamines have some species-specific effects. There is evidence to suggest that the pacemaker current is brought about by the activation of non-selective channels ($i_r$), which are permeable to Na$^+$ [4, 5] rather than the inactivation of K$^+$ channels ($i_{k_2}$) [40]. Consistent with this idea is the recently reported increase of $a_{Na}$ concomitant with membrane depolarization induces by noradrenaline in quiescent cardiac Purkinje fibers from sheep [40]. Moreover, another study has shown that isoproterenol induced depolarization in resting potential by activating $i_r$, despite an absence of change in $a_{Na}$ [39]. It is possible that the forskolin-induced depolarization in the quiescent fibers is brought about by the same mechanism responsible for the depolarization induced by catecholamines. The less pronounced or lack of effect of forskolin on membrane potential in the presence of strophanthidin or 0 mM [K$^+$]o may thereby be due to the membrane depolarization under these circumstances. A depolarization of membrane potential would shift the voltage dependence of $i_r$ and diminish the effect of forskolin on membrane potential. If Na$^+$ entry is at least partially responsible for the depolarization of resting potential induced by forskolin, then more Na$^+$ must be removed from the cytosol than that enters the cytosol in order for $a_{Na}$ to decrease. The entry of one Na$^+$ through a channel results in a net gain of one positive charge in the cytosol, while it takes the removal of three Na$^+$ by the Na$^+$ pump to subtract one positive charge from the cytosol because the stoichiometry for Na$^+$ pump is 3 Na$^+$:2 K$^+$. Therefore, provided that the activity of the Na$^+$ pump can be enhanced directly by forskolin sufficiently enough (rather than indirectly via an increase in Na$^+$ influx), it is possible for $a_{Na}$ to decrease in spite of a depolarization caused by the entry of Na$^+$. The other possibility for forskolin-induced depolarization is a decrease in membrane permeability to K$^+$. The other factor that may account for the change in $a_{Na}$ seen in the present study is the effect of membrane potential on Na$^+$ leak or Na–Ca exchange. However, this factor cannot account for all the changes in $a_{Na}$ because forskolin depolarized the resting membrane potential in the presence of strophanthidin but did not decrease $a_{Na}$. Recently, evidence has been presented that indicates some dependency of $a_{Na}'$ on membrane potential [8,12]. Depolarization caused $a_{Na}$ to decrease and hyperpolarization caused $a_{Na}$ to increase. In light of this evidence, it is possible that some of the forskolin-induced decrease in $a_{Na}$ may be directly related to the depolarization in membrane potential. As for Na–Ca exchange with a stoichiometry of three or more Na$^+$ for 1 Ca$^{2+}$, depolarization around resting membrane potential would favor less Na$^+$ influx, which would lead to a decrease in $a_{Na}$. Moreover, it has been shown that isoproterenol decreases [Ca$^{2+}$i] by about 20 nm in normal polarized cardiac myocytes [33]. This decrease in [Ca$^{2+}$], would then slow down Na$^{+}$-dependent Ca$^{2+}$ efflux and lead to a reduction in $a_{Na}'$. Note that Na$^{+}$-sensitive microelectrodes were also sensitive to Ca$^{2+}$ with a selectivity coefficient ($k_{NaCa}$) of 2.5 [30].
The changes in \([\text{Ca}^{2+}]_i\) of 20 nM would contribute to the apparent change in \(a_{\text{Na}}^i\) by 0.25 nM. Therefore, the average change of 2.7 mM of \(a_{\text{Na}}^i\) in quiescent fibers was overestimated.

The prevention of the decrease in \(a_{\text{Na}}^i\) by strophanthidin and K\(+\)-free solutions is identical to the observations in recent reports \cite{15,20}. However, an unequivocal conclusion that forskolin directly stimulates the Na\(^+\) pump cannot be derived from the present experiments. This is because: (A) \(a_{\text{Na}}^i\) was already rather high (about 20 mM) when forskolin was applied after strophanthidin or K\(+\)-free solutions. A 1 to 2 mM change in \(a_{\text{Na}}^i\) would produce only a 1 mV voltage deflection under this condition and, thus, would make it difficult to detect. (B) The high \(a_{\text{Na}}^i\), which leads to high \(a_{\text{Ca}}^i\), may have altered the process that regulates \(a_{\text{Na}}^i\) and biased the effect of forskolin. (C) The membrane potential was depolarized in the presence of both strophanthidin and 0 mM \([\text{K}^+]_0\). This depolarization in membrane potential induced under these two conditions may also influence the action of forskolin on \(a_{\text{Na}}^i\). Therefore, the decrease in \(a_{\text{Na}}^i\) by forskolin is associated with the state of Na\(^+\) pump, however, a direct stimulation of the Na\(^+\) pump by forskolin cannot be established in the present study.

Prolonged exposure to strophanthidin caused small oscillations in membrane potential (0 to 3 mV) at the level near the resting membrane potential. It has been shown that the current associated with these oscillations is activated by the oscillatory Ca\(^{2+}\) release from Ca\(^{2+}\) overloaded sarcoplasmic reticulum \cite{14,18,27,45}. It would seem likely that forskolin increases oscillations of intracellular calcium by increasing Ca\(^{2+}\) loading in sarcoplasmic reticulum as similar to isoproterenol \cite{17}.

The results showing that forskolin, like catecholamines and cAMP analogues \cite{20,41,42}, has a potent positive inotropic effect are consistent with the findings of other investigators \cite{26,34}. It was discovered that forskolin increased the slow inward calcium current, and that the increase in this current was directly related to an increase in twitch tension \cite{39}. An increase in the slow inward calcium current would possibly account for the elevation of the plateau phase of the action potential \cite{23,24,35}. However, one should be cautious in relating the plateau of action potential with slow inward calcium current since other currents also determine the plateau of action potential \cite{13}.

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