

Original Article

Isoform-specific regulation of the Na⁺-K⁺ pump by adenosine in guinea pig ventricular myocytes

Zhe ZHANG, Hui-cai GUO, Li-nan ZHANG, Yong-li WANG*

Department of Pharmacology, Hebei Medical University, Shijiazhuang 050017, China

Aim: The present study investigated the effect of adenosine on Na⁺-K⁺ pumps in acutely isolated guinea pig (*Cavia* sp.) ventricular myocytes.

Methods: The whole-cell, patch-clamp technique was used to record the Na⁺-K⁺ pump current (I_p) in acutely isolated guinea pig ventricular myocytes.

Results: Adenosine inhibited the high DHO-affinity pump current (I_h) in a concentration-dependent manner, which was blocked by the selective adenosine A₁ receptor antagonist DPCPX and the general protein kinase C (PKC) antagonists staurosporine, GF 109203X or the specific δ isoform antagonist rottlerin. In addition, the inhibitory action of adenosine was mimicked by a selective A₁ receptor agonist CCPA and a specific activator peptide of PKC- δ , PP114. In contrast, the selective A_{2A} receptor agonist CGS21680 and A₃ receptor agonist Cl-IB-MECA did not affect I_h . Application of the selective A_{2A} receptor antagonist SCH58261 and A₃ receptor antagonist MRS1191 also failed to block the effect of adenosine. Furthermore, H89, a selective protein kinase A (PKA) antagonist, did not exert any effect on adenosine-induced I_h inhibition.

Conclusion: The present study provides the electrophysiological evidence that adenosine can induce significant inhibition of I_h via adenosine A₁ receptors and the PKC- δ isoform.

Keywords: Na⁺-K⁺ pump; isoform; regulation; adenosine; patch-clamp techniques; protein kinase C

Acta Pharmacologica Sinica (2009) 30: 404–412; doi: 10.1038/aps.2009.26; published online 23rd March 2009

Introduction

The Na⁺-K⁺ pump is a ubiquitous plasma membrane-bound enzyme that transports three Na⁺ for every two K⁺ into the cell by hydrolyzing ATP. Functional Na⁺-K⁺ pumps contain a catalytic α -subunit and a glycosylated β -subunit. The α -subunit alone binds Na⁺, K⁺, ATP, and cardiac glycosides. At present, four isoforms of the α -subunit (α_1 – α_4) have been identified, and each has a unique tissue distribution^[1,2]. In guinea pig (*Cavia* sp.) ventricular myocytes, only the α_1 - and α_2 -isoforms, which correspond to the low- and high-affinity isoforms for cardiac glycosides, respectively, are expressed^[3,4].

The Na⁺-K⁺ pump is subject to regulation by a variety of hormones or transmitters, including catecholamines, aldosterone, insulin, angiotensin, thyroid hormone and adenosine^[5–9]. For instance, Alzamora *et al*^[5] demonstrated

that aldosterone has a non-genomic effect on the Na⁺-K⁺ pump of vascular tissue, which is mediated by PKC activation. In patch-clamped guinea pig ventricular myocytes, Gao *et al*^[4] showed that the high DHO-affinity pump current (I_h) is regulated by α -adrenergic agonists via a PKC-dependent pathway, whereas the low DHO-affinity pump current (I_l) is regulated by β -adrenergic agonists via a PKA-dependent pathway.

Adenosine, a purine nucleoside, is widely distributed in all tissues and body fluids. It is well known that adenosine exerts its cardiovascular effects by interacting with four types of G-protein coupled receptors (A₁R, A_{2A}R, A_{2B}R, A₃R)^[10]. Considering that the regulatory effect of adenosine is a receptor-mediated process that involves the activation of PKA and PKC^[10–12], one would expect to find a potential regulatory effect of adenosine on the Na⁺-K⁺ pump. Indeed, several recent studies have been carried out to address this issue. However, these studies have yielded disparate results. Caruso-Neves *et al*^[6] demonstrated that in the Malpighian tubule cells of the blood-sucking bug *Rhodnius prolixus*,

* Correspondence to Prof Yong-li WANG.

E-mail wangyongli@gmail.com

Received 2008-08-14 Accepted 2009-02-23

adenosine inhibited the $\text{Na}^+\text{-K}^+$ pump through interaction with the A_1R . Krumschnabel *et al*^[13] have also reported adenosine A_1R mediated $\text{Na}^+\text{-K}^+$ pump inhibition in the hepatocytes of goldfish (*Carassius auratus*) and trout (*Oncorhynchus mykiss*). These findings together suggest that the A_1R may play a role in adenosine-induced $\text{Na}^+\text{-K}^+$ pump inhibition. In contrast, Darlington *et al*^[14] found an ultra-filtrate in plasma from mammalian species (dog, rat, calf) that could stimulate the $\text{Na}^+\text{-K}^+$ pump activity. They identified the stimulator to be adenosine and suggested that the effect of adenosine on the $\text{Na}^+\text{-K}^+$ pump was independent of adenosine receptors.

Given the lack of uniformity of these observations described above, the present study was designed to ascertain the effects of adenosine on the $\text{Na}^+\text{-K}^+$ pump in guinea pig ventricular myocytes and to further clarify the potential underlying mechanisms involved.

Materials and methods

Preparation of single ventricular myocytes Adult guinea pigs (250–350 g) were purchased from Hebei Medical University Laboratorial Animal Center (Shijiazhuang, China). Single ventricular myocytes were enzymatically isolated as described in Gao *et al*^[15] with minor modifications. Briefly, hearts from anesthetized (sodium pentobarbitone, 120 mg/kg, ip) guinea pigs were excised quickly and perfused retrogradely through the aorta (about 8 mL/min) with oxygenated Ca^{2+} -free Tyrode's solution (mmol/L): 137.7 NaCl, 2.3 NaOH, 5.4 KCl, 1 MgCl_2 , 5 HEPES, and 10 glucose (pH adjusted to 7.4 with NaOH) at 37 °C. After the perfusate was free of blood, the solution was changed to Ca^{2+} -free Tyrode's solution containing 12 mg/mL collagenase (Serva, Heidelberg, Germany) for 10 min. Digested ventricles were thereafter cut into small species and agitated mechanically in high- K^+ Kraft-Brühe (KB) solution to obtain single ventricular myocytes. The composition of the KB solution was (mmol/L): 83 KCl, 30 K_2HPO_4 , 5 MgSO_4 , 2 KOH, 5 sodium pyruvic acid, 5 β -OH-butyric acid, 5 creatine, 20 taurine, 10 glucose, 0.5 EGTA, 5 HEPES, and 5 $\text{Na}_2\text{-ATP}$ (pH adjusted to 7.2 with KOH). The dissociated cells were then kept in KB solution at room temperature for at least 1 h before the experiment.

Electrophysiology Cells were placed in the 0.3 mL superfusion chamber mounted on the stage of an inverted microscope (Nikon TE2000-S), allowed to attach to its glass bottom, and then superfused with the extracellular solution containing (mmol/L): 137.7 NaCl, 2.3 NaOH, 5.4 KCl, 1 MgCl_2 , 5 HEPES, 10 glucose, 2 BaCl_2 and 1 CdCl_2 (pH adjusted to 7.4 with NaOH). The chamber was perfused

at a rate of about 2 mL/min and the solution exchange was complete within 2 min. The holding current was recorded using a whole-cell, patch-clamp technique and amplified using an Axopatch 700B amplifier (Axon Instruments). The sampling rate was 200 ms/point, and the data were low-pass filtered at 2 Hz. Patch electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instruments) and fire-polished to a final resistance of 1–3 M Ω when filled with the standard pipette solution, which contained (mmol/L) 50 sodium aspartic acid, 20 potassium aspartic acid, 30 CsOH, 20 TEACl, 5 MgSO_4 , 5 HEPES, 11 EGTA, 10 glucose, 5 $\text{Na}_2\text{-ATP}$, 1 CaCl_2 (pH adjusted to 7.2 with CsOH). Solutions were designed to minimize all other components of membrane current (K^+ currents were blocked by replacing pipette K^+ with Cs^+ and TEA^+ and adding Ba^{2+} to the extracellular solution; Ca^{2+} channel and $\text{Na}^+\text{-Ca}^{2+}$ exchanger currents were inhibited by including 1 mmol/L CdCl_2 in the extracellular solution). Under these conditions, I_p was defined as the difference in currents before and after the addition of DHO, a specific and reversible inhibitor of the $\text{Na}^+\text{-K}^+$ pump. We voltage-clamped the myocytes to 0 mV, a saturating voltage for the $\text{Na}^+\text{-K}^+$ pump. For each experiment, both control and test I_p were obtained from the same cell to avoid cell-to-cell variability. For measurement of the voltage dependence of I_p , a voltage-ramp protocol going from +20 to -100 mV in a 4-s period was used in some experiments. This protocol was applied to each myocyte at least three times: (1) under control conditions, (2) during the adenosine effect on I_p and (3) after the $\text{Na}^+\text{-K}^+$ pump was blocked with DHO. The I_p values were obtained by digital subtraction of the membrane current in the presence of DHO from that in its absence at each test potential. All recordings were carried out at room temperature (22–25 °C) and data acquisition was achieved using pClamp 9.0 software.

Drugs The drugs used in these studies and their abbreviations include CCPA, DPCPX, CGS-21680, Cl-IB-MECA, staurosporine (St), H89, adenosine (Ado), GF 109203X, MRS1191, dihydroouabain (DHO), Gö-6976, rottlerin, PP114, and SCH58261. All chemical reagents were purchased from Sigma Chemical Co (St Louis, MO, USA). Adenosine and DHO were dissolved in deionized water, and all other chemicals were dissolved in dimethylsulfoxide (DMSO) to prepare stock solutions that were stored at -20 °C. The final concentration of DMSO never exceeded 0.1%, which produced no detectable effect on I_p .

Data analysis and statistics The data were analyzed with Clampfit 9.0 (Axon Instruments) and Origin 7.0 (Originlab Corporation) software. All values are presented as means \pm SEM. Statistical analysis of differences between

two groups was carried out using Student's paired *t*-test. Two-way ANOVA was performed to determine significance between the voltage dependence curves. A value of $P < 0.05$ was considered statistically significant.

Results

Adenosine specifically inhibits I_h in guinea pig ventricular myocytes I_1 (α_1 -isoform related I_p) and I_h (α_2 -isoform related I_p) are distinguishable by their different sensitivities to cardiac glycosides, with 5 $\mu\text{mol/L}$ DHO blocking I_h and 1 mmol/L DHO blocking I_1 ^[3,16]. Experiments were carried out to determine the effects of adenosine on each of these

two isoforms.

First, we examined the effect of adenosine on I_h . Figure 1A illustrates that a physiological concentration of adenosine (1 nmol/L)^[17,18] decreased I_h from 16.1 ± 0.3 pA ($I_{h(\text{Con})}$) to 9.9 ± 0.7 pA ($I_{h(\text{Ado})}$) by 39% ($n=8$, $P < 0.05$, upper panel). The decrease in I_p was not due to pump "run-down" because the adenosine effect was reversible upon washout (lower panel of Figure 1A). Figure 1B shows that adenosine inhibited I_h in a concentration-dependent manner from 1×10^{-11} to 1×10^{-5} mol/L (8% to 47%). Adenosine (1×10^{-8} mol/L) caused maximal inhibition. No significant effect was observed at concentrations of adenosine below 1×10^{-11} mol/L. The mean data from five to seven

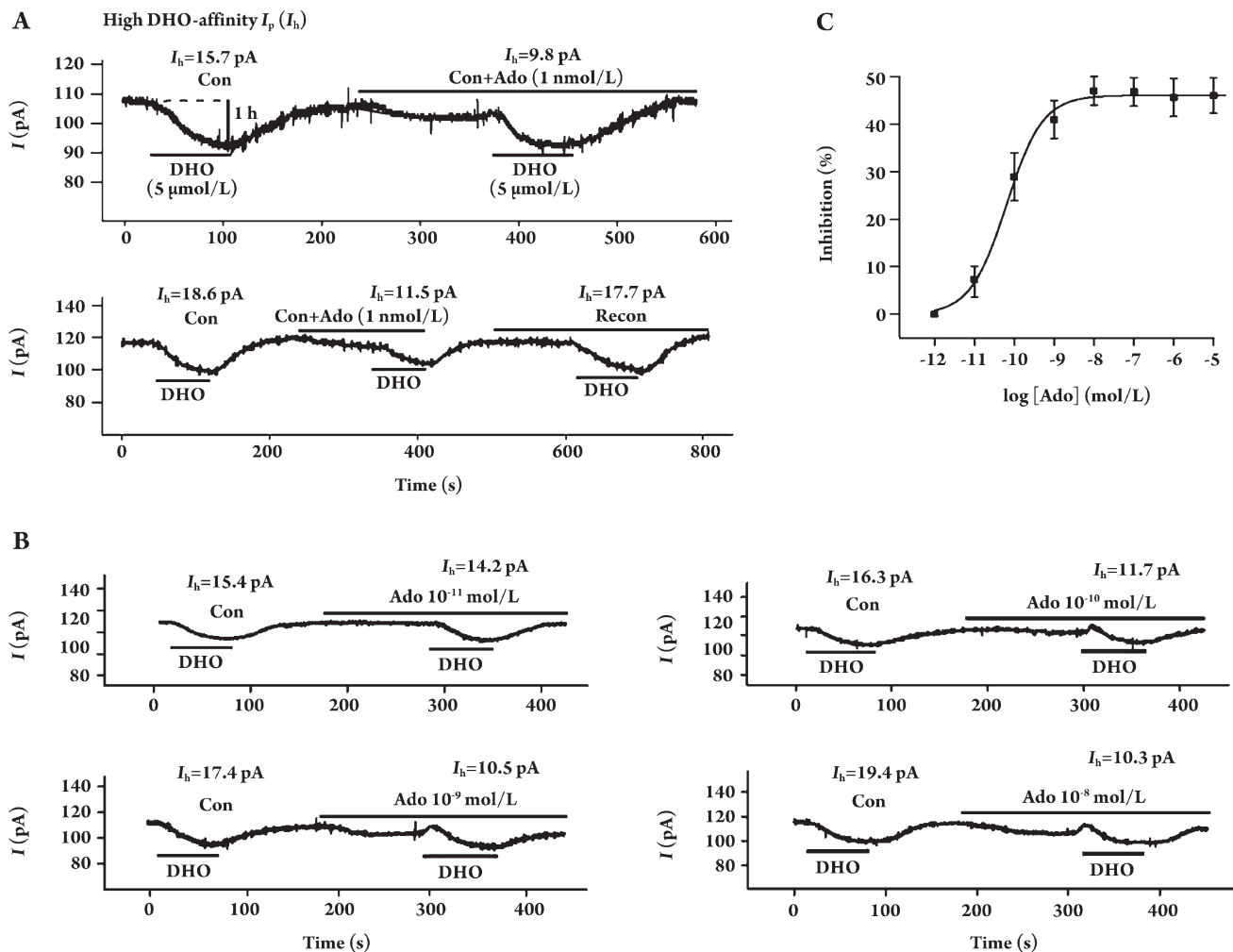


Figure 1. Adenosine inhibits I_h in guinea pig ventricular myocytes. (A) Upper panel: a typical trace showing the effect of 1 nmol/L adenosine on I_h . The lower and upper horizontal lines indicate the application of 5 $\mu\text{mol/L}$ DHO and an adenosine-containing solution, respectively. The vertical bar illustrates the measured I_h amplitude. Lower panel: a typical trace showing that the adenosine effects on I_h were not due to pump "run-down". (B) Representative traces of the effect of adenosine (1×10^{-11} – 1×10^{-5} mol/L) on I_h . (C) The percentage inhibition of I_h was plotted for each concentration of adenosine used. The error bars indicate means \pm SEM.

cells are shown in Figure 1C, where the percentage inhibition of I_h is plotted against the concentration of adenosine.

Next, we investigated the effect of adenosine on I_i . The entire experiment was performed in the presence of 5 $\mu\text{mol/L}$ DHO to block I_h , and I_i was measured following the application of 1 mmol/L DHO. Figure 2A shows that adenosine did not change I_i significantly [113.8 \pm 0.9 pA for $I_{i(\text{Ado})}$, $P>0.05$ vs 115.4 \pm 0.7 pA for $I_{i(\text{Con})}$, $n=7$]. In addition, increasing adenosine to 10 $\mu\text{mol/L}$ had no effect on I_i [91.2 \pm 0.8 pA for $I_{i(\text{Ado})}$, $P>0.05$ vs 94.4 \pm 0.9 pA for $I_{i(\text{Con})}$, $n=10$, Figure 2B]. These results indicate that the inhibitory effect of adenosine on the $\text{Na}^+\text{-K}^+$ pump current is specifically mediated via the $\text{Na}^+\text{-K}^+$ pump α_2 -isoform, so our subsequent studies were focused primarily on the effect of adenosine on I_h .

The adenosine-induced inhibition of I_h is voltage independent Because I_p in guinea pig ventricular myocytes is voltage dependent^[16], we went further to examine the effect of adenosine on the I_h - V_m relationship. Figure 3A shows the voltage-ramp protocol applied to the myocytes. The relationships were normalized to the I_h recorded at 0 mV to facilitate comparison of their slopes, which are summarized in Figure 3B. The normalized I_h was generally lower in the presence of adenosine than that in its absence. The difference between these slopes was not statistically significant ($n=6$, $P>0.05$; two-way ANOVA). Thus, adenosine-induced I_h inhibition is voltage independent.

Inhibition of I_h by adenosine is mediated by A_1 receptors All four adenosine receptor subtypes are expressed in guinea pig ventricular myocytes, among which A_1 R is consid-

ered predominant^[19,20]. Thus, we tested whether adenosine inhibition of I_h was mediated by A_1 R. Figure 4A indicates that DPCPX (10 nmol/L), a selective A_1 R antagonist, had no effect on I_h by itself (upper panel), but completely abolished adenosine-induced I_h inhibition [7.8 \pm 0.5 pA for $I_{h(\text{Ado})}$, $P<0.05$ vs 14.7 \pm 0.3 pA for $I_{h(\text{Con})}$, and 14.1 \pm 0.6 pA for $I_{h(\text{Ado+DPCPX})}$, $P>0.05$ vs 14.7 \pm 0.3 pA for $I_{h(\text{Con})}$, $n=8$, lower panel]. In addition, CCPA (10 nmol/L), a selective agonist for A_1 R, produced a marked inhibition of I_h [7.8 \pm 0.4 pA for $I_{h(\text{CCPA})}$, $P<0.05$ vs 15.7 \pm 0.3 pA for $I_{h(\text{Con})}$, $n=9$, Figure 4B]. In addition, the CCPA effect was absent in the presence of DPCPX (data not shown). These results strongly suggest that the effect of adenosine was the result of stimulation of the adenosine A_1 R.

To investigate the possible participation of other adenosine receptor subtypes, the A_{2A} R and A_3 R selective agonists CGS21680 and Cl-IB-MECA, respectively, were tested. As shown in Figure 4C, CGS21680 (0.2 $\mu\text{mol/L}$) had no significant effect on I_h [16.0 \pm 0.4 pA for $I_{h(\text{CGS21680})}$, $P>0.05$ vs 16.2 \pm 0.7 pA for $I_{h(\text{Con})}$, $n=9$]. Similarly, Cl-IB-MECA (0.5 $\mu\text{mol/L}$) had no effect on I_h [18.2 \pm 0.5 pA for $I_{h(\text{Cl-IB-MECA})}$, $P>0.05$ vs 18.5 \pm 0.6 pA for $I_{h(\text{Con})}$, $n=9$, Figure 4D]. We also observed that perfusion of adenosine, together with SCH58261 and MRS1191 (0.1 $\mu\text{mol/L}$ each), the A_{2A} R and A_3 R selective antagonists, respectively, did not alter the adenosine effect on I_h [11.8 \pm 0.6 pA for $I_{h(\text{Ado})}$, $P<0.05$ vs 22.1 \pm 0.5 pA for $I_{h(\text{Con})}$, and 10.3 \pm 0.8 pA for $I_{h(\text{Ado+SCH58261+MRS1191})}$, $P<0.05$ vs 22.1 \pm 0.5 pA for $I_{h(\text{Con})}$, $n=9$] (Figure 4E). It is, therefore, highly unlikely that A_{2A} R and

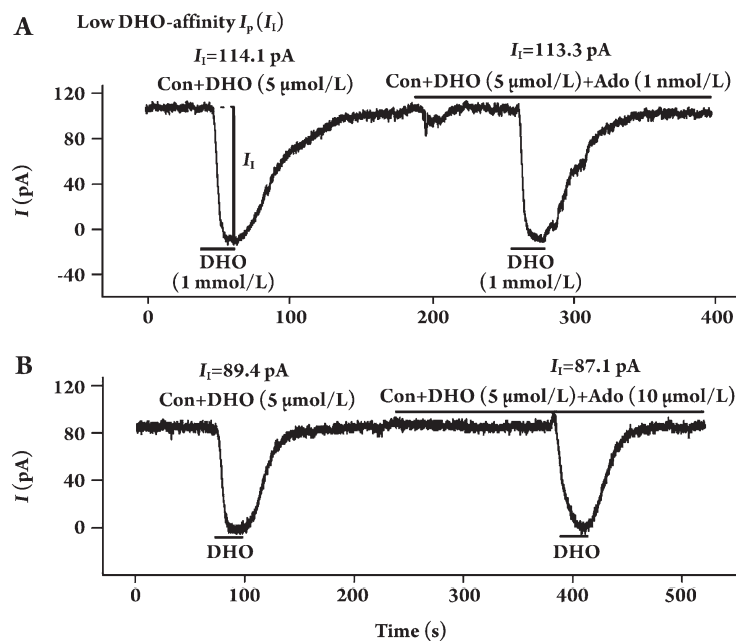


Figure 2. Adenosine has no effect on I_i in guinea pig ventricular myocytes. (A) A typical trace showing the effect of 1 nmol/L adenosine on I_i . The vertical bar illustrates the measured I_i amplitude. (B) A typical trace showing the effect of 10 $\mu\text{mol/L}$ adenosine on I_i .

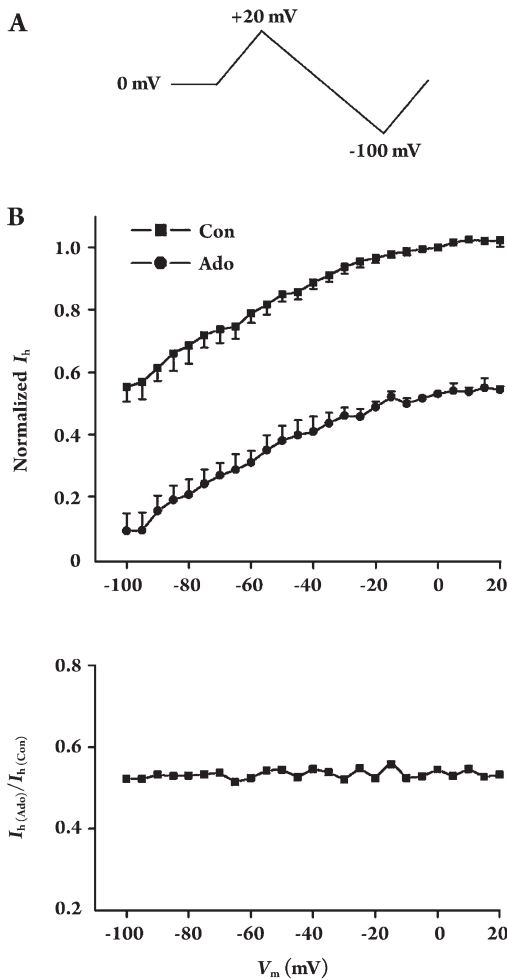


Figure 3. The voltage dependence of I_h is not shifted by adenosine. (A) Voltage-ramp protocol applied to myocytes. (B) Normalized current-voltage relationship of I_h in the presence (●) and absence (■) of adenosine. The error bars indicate means \pm SEM. The lower panel graphs the ratio of $I_{h(Ado)}/I_{h(Con)}$.

A_3R are involved in adenosine-induced inhibition of I_h .

The activation of PKC- δ inhibits I_h PKC is stimulated by A_1R activation in ventricular myocytes^[11, 21]. Therefore, we examined the effect of adenosine in the presence of staurosporine, a well-characterized PKC inhibitor. Perfusion of 1.5 μ mol/L staurosporine alone did not modify I_h (data not shown) but completely abolished adenosine-induced I_h inhibition [10.5 \pm 0.6 pA for $I_{h(Ado)}$, $P < 0.05$ vs 19.8 \pm 0.5 pA for $I_{h(Con)}$, and 18.8 \pm 0.9 pA for $I_{h(Ado+St)}$, $P > 0.05$ vs 19.8 \pm 0.5 pA for $I_{h(Con)}$, $n = 10$, upper panel of Figure 5A]. Similar results were also obtained with GF 109203X, a highly specific PKC inhibitor. Adenosine did not inhibit I_h in the presence of 1 μ mol/L GF 109203X [11.9 \pm 0.6 pA for $I_{h(Ado)}$, $P < 0.05$ vs 22.4 \pm 0.8 pA for $I_{h(Con)}$, and 20.3 \pm 0.9 pA for $I_{h(Ado+GF 109203X)}$,

$P > 0.05$ vs 22.4 \pm 0.8 pA for $I_{h(Con)}$, $n = 8$, lower panel of Figure 5A]. This reinforces the view that adenosine inhibits I_h through a PKC-dependent mechanism.

Because adult cardiomyocytes express multiple PKC isoforms^[22], we asked which one might be involved. We initially explored the role of the classical PKC isoforms (PKC- α and β) using the inhibitor Gö-6976. The upper panel of Figure 5B shows that adenosine still inhibited I_h in the presence of 100 nmol/L Gö-6976 [14.5 \pm 0.6 pA for $I_{h(Ado)}$, $P < 0.05$ vs 27.4 \pm 0.8 pA for $I_{h(Con)}$, and 12.8 \pm 0.9 pA for $I_{h(Ado+Gö-6976)}$, $P < 0.05$ vs 27.4 \pm 0.8 pA for $I_{h(Con)}$, $n = 8$]. This indicates that classical PKC isoforms are not involved. Given the prominent role of PKC- δ during A_1R activation by adenosine^[23, 24], we next examined the effect of rottlerin, a specific PKC- δ inhibitor, on the adenosine effect. Inhibition of PKC- δ with 10 μ mol/L rottlerin completely abolished the effect of adenosine [12.9 \pm 0.6 pA for $I_{h(Ado)}$, $P < 0.05$ vs 24.4 \pm 0.8 pA for $I_{h(Con)}$, and 22.3 \pm 0.8 pA for $I_{h(Ado+rottlerin)}$, $P > 0.05$ vs 24.4 \pm 0.8 pA for $I_{h(Con)}$, $n = 8$, middle panel of Figure 5B], indicating that PKC- δ is required for the adenosine effect on I_h . To confirm this, a specific activator peptide of PKC- δ , PP114, was used. Similar to adenosine, PP114 (200 nmol/L) caused a marked decrease in I_h [10.8 \pm 0.7 pA for $I_{h(PP114)}$, $P < 0.05$ vs 22.7 \pm 0.6 pA for $I_{h(Con)}$, $n = 9$, lower panel of Figure 5B]. The PP114 effect was also blocked by GF 109203X or rottlerin (data not shown). Taken together, these results indicate that PKC- δ plays a crucial role in the inhibition of I_h by adenosine.

To exclude the possible involvement of PKA in the adenosine-mediated I_h inhibition, the effect of adenosine was examined in the presence of H89, an inhibitor of PKA. Figure 5C shows that inhibition of PKA with 1 μ mol/L H89 failed to abolish the effect of adenosine on I_h [11.0 \pm 0.6 pA for $I_{h(Ado)}$, $P < 0.05$ vs 20.7 \pm 0.4 pA for $I_{h(Con)}$, and 10.9 \pm 0.8 pA for $I_{h(Ado+H89)}$, $P < 0.05$ vs 20.7 \pm 0.4 pA for $I_{h(Con)}$, $n = 8$]. Thus, PKA appears not to mediate I_h inhibition by adenosine.

Discussion

Numerous studies over the years have highlighted the isoform-specific modulation of Na^+ - K^+ pumps by transmitters or kinases in many types of cells^[4, 25, 26]. In this study, we found that the α_2 -isoform Na^+ - K^+ pumps are specifically inhibited by adenosine. This implies a link among a specific adenosine receptor, its associated kinases, and inhibition of I_h in ventricular myocytes. In light of this, the goal of this study was to identify potential mechanisms by which adenosine exerts its effects on I_h .

We first examined which type of adenosine receptor is involved in this phenomenon. Considering that the adenosine

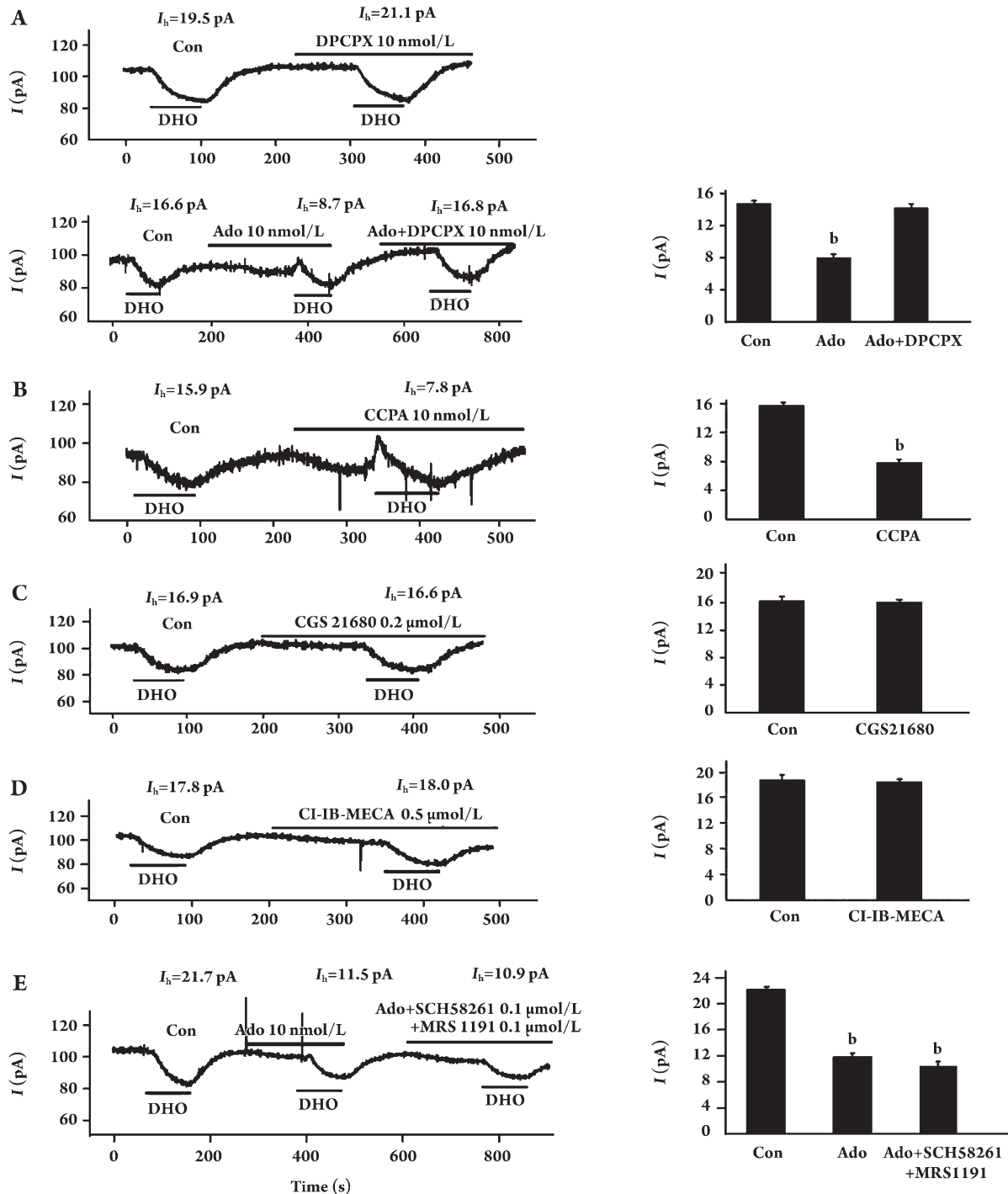


Figure 4. Adenosine-induced inhibition of I_h is mediated by adenosine A_1R , but not by $A_{2A}R$ or A_3R . (A) Upper panel: a typical trace showing the effect of the A_1R selective antagonist DPCPX (10 nmol/L) alone on I_h . Lower panel: a typical trace in the left panel showing the effect of adenosine on I_h in the presence and absence of DPCPX (10 nmol/L). The right panel shows a summary of the results. (B) A typical trace in the left panel showing the effect of the A_1R selective agonist CCPA (10 nmol/L) on I_h . The right panel shows a summary of the results. (C, D) Typical traces in the left panels showing the effects of the $A_{2A}R$ and A_3R selective agonists CGS21680 (0.2 μ mol/L) and CI-IB-MECA (0.5 μ mol/L), respectively, on I_h . The right panels show summaries of the results. (E) A typical trace in the left panel showing the effect of adenosine on I_h in the presence and absence of the $A_{2A}R$ and A_3R selective antagonists SCH58261 and MRS1191 (0.1 μ mol/L each). The right panel shows a summary of the results. The error bars indicate means \pm SEM. ^b P <0.05 vs controls.

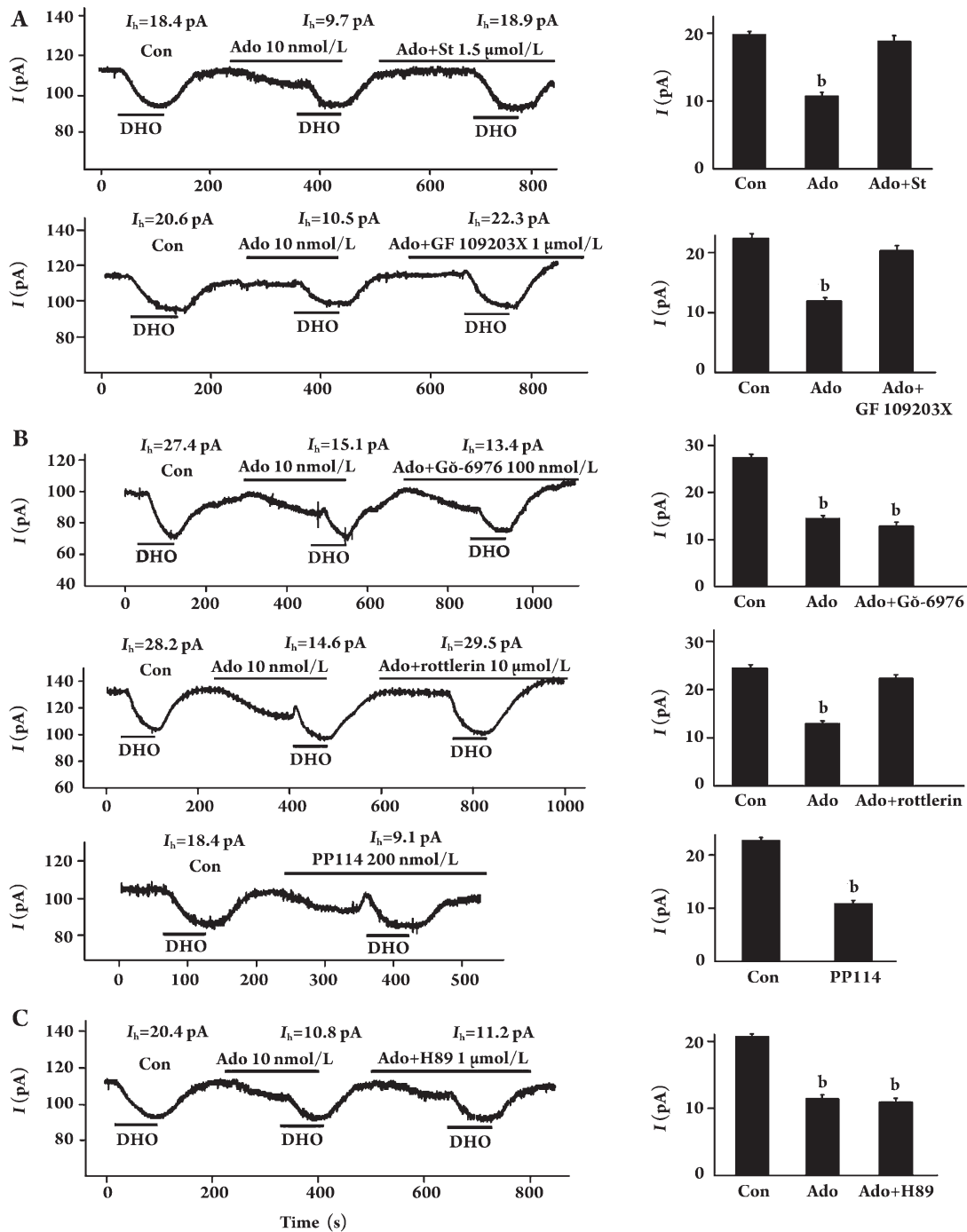


Figure 5. PKC- δ is primarily involved in the inhibitory effect of adenosine on I_h , whereas PKA is not involved. (A) Typical traces in the left panels showing the effects of adenosine on I_h in the presence and absence of the general PKC antagonists St (1.5 μ mol/L, upper panel) or GF 109203X (1 μ mol/L, lower panel). The right panels show summaries of the results. (B) Upper panel: a typical trace in the left panel showing the effect of adenosine on I_h in the presence and absence of the PKC- α and β inhibitor Gö-6976 (100 nmol/L). The right panel shows a summary of the results. Middle panel: a typical trace in the left panel showing the effect of adenosine on I_h in the presence and absence of the PKC- δ inhibitor rottlerin (10 μ mol/L). The right panel shows a summary of the results. Lower panel: a typical trace in the left panel showing the effect of the PKC- δ activator PP114 (200 nmol/L) on I_h . The right panel shows a summary of the results. (C) A typical trace in the left panel showing the effect of adenosine on I_h in the presence and absence of the PKA antagonist H89 (1 μ mol/L). The right panel shows a summary of the results. The error bars indicate means \pm SEM. ^b P <0.05 vs controls.

ine concentration we used is close to that described for the high-affinity A_1R (0.5–100 nmol/L)^[27], we speculate that the inhibitory effects of adenosine on the Na^+K^+ pump are most likely mediated via the A_1R . Indeed, using selective AR agonists and antagonists, we have demonstrated a specific role for A_1R in adenosine-mediated I_h inhibition, whereas $A_{2A}R$ and A_3R are not involved, in accordance with earlier studies using *R prolixus* and *C auratus*^[6, 13]. There are two possible explanations for the above results. First, the $A_{2A}R$ is coupled to the cAMP-PKA pathway^[28, 29], which is targeted to the α_1 -isoform of the Na^+K^+ pump^[4]. Hence, $A_{2A}R$ activation could not lead to any change in I_h . A second possibility that may be pertinent to our results is the absence of functional $A_{2A}R$ or A_3R proteins in cardiac myocytes^[10, 30]. These two lines of evidence completely rule out the involvement of $A_{2A}R$ and A_3R in the present study.

We next examined the possible mechanism(s) underlying I_h inhibition by A_1R stimulation. Binding of adenosine to A_1R inhibits adenylyl cyclase and stimulates PKC via activation of the pertussis toxin sensitive G proteins G_i and/or G_o ^[28, 29]. Using selective antagonists for PKC and PKA, we observed that the adenosine A_1R triggers the PKC pathway to inhibit I_h , but the cAMP-PKA pathway is not involved. Specifically, this inhibition is predominantly mediated by the novel PKC- δ isoform. Our results are consistent with those of Gao *et al*^[4], who demonstrated that I_h was specifically regulated by PKC. However, in their study, I_h was increased by α -adrenoceptor stimulation via the PKC pathway, which is not congruent with our result showing a PKC- δ -mediated decrease in I_h . The most likely explanation for this discrepancy is the stimulation of different PKC isoforms by α -adrenoceptor activation. Indeed, we observed that α -adrenoceptor activation increases I_h in a PKC- β dependent manner using the inhibitor LY333531, which substantiates our results (data not shown). Taken together, these observations strongly suggest that adenosine-induced I_h inhibition is mediated by the PKC- δ isoform. The exact mechanisms for PKC- δ -mediated inhibition of I_h require further study, however, it most likely involves a phosphorylation-dependent process. In this case, PKC- δ may directly phosphorylate the pump protein to induce conformational changes, thus decreasing the turnover rate of each pump^[1, 31]. In addition, the recent observation that phospholemman (FXVD1) associates with the cardiac Na^+K^+ pump^[32] offers another subunit that may confer sensitivity to PKC- δ .

In conclusion, the major findings are that adenosine inhibits I_h via activation of A_1R and PKC- δ . This finding may have implications for our understanding of the antiarrhythmic effect of adenosine when used clinically. The inhibition

of Na^+K^+ pump prolongs action potential duration and myocardial refractoriness, which is involved in the mechanisms of two other widely used antiarrhythmic agents, bretylium and amiodarone^[33, 34]. Thus, it is tempting to speculate that the antiarrhythmic effects of adenosine are in part caused by Na^+K^+ pump inhibition.

Acknowledgements

This project was supported by the Natural Science Foundation of Hebei Province (No 30200030).

Author contribution

Yong-li WANG and Zhe ZHANG designed research; Zhe ZHANG and Hui-cai GUO performed research; Zhe ZHANG and Li-nan ZHANG analyzed data; Zhe ZHANG and Yong-li WANG wrote the paper.

References

- 1 Blanco G, Mercer RW. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* 1998; 275: F633–50.
- 2 Lopina OD. Na^+ , K^+ -ATPase: structure, mechanism, and regulation. *Membr Cell Biol* 2000; 13: 721–44.
- 3 Gao J, Mathias RT, Cohen IS, Baldo GJ. Two functionally different Na/K pumps in cardiac ventricular myocytes. *J Gen Physiol* 1995; 106: 995–1030.
- 4 Gao J, Wymore R, Wymore RT, Wang Y, McKinnon D, Dixon JE, *et al*. Isoform-specific regulation of the sodium pump by alpha- and beta-adrenergic agonists in the guinea-pig ventricle. *J Physiol* 1999; 516 (Pt 2): 377–83.
- 5 Alzamora R, Marusic E, Gonzalez M, Michea L. Nongenomic effect of aldosterone on Na^+ , K^+ -adenosine triphosphatase in arterial vessels. *Endocrinology* 2003; 144: 1266–72.
- 6 Caruso-Neves C, Monteiro SO, de Oliveira CF, Filho CC, Lopes AG. Adenosine modulates the Na^+/K^+ ATPase activity in malpighian tubules isolated from *Rhodnius prolixus*. *Arch Insect Biochem Physiol* 2000; 43: 72–7.
- 7 Hakam AC, Hussain T. Angiotensin II AT_2 receptors inhibit proximal tubular Na^+K^+ -ATPase activity via a NO/cGMP-dependent pathway. *Am J Physiol Renal Physiol* 2006; 290: F1430–6.
- 8 Hansen PS, Buhagiar KA, Gray DF, Rasmussen HH. Voltage-dependent stimulation of the Na^+K^+ pump by insulin in rabbit cardiac myocytes. *Am J Physiol Cell Physiol* 2000; 278: C546–53.
- 9 Sharabani-Yosef O, Nir U, Sampson SR. Thyroid hormone up-regulates Na^+/K^+ pump alpha2 mRNA but not alpha2 protein isoform in cultured skeletal muscle. *Biochim Biophys Acta* 2002; 1573: 183–8.
- 10 Peart JN, Headrick JP. Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacol Ther* 2007; 114: 208–21.
- 11 Miura T, Liu Y, Kita H, Ogawa T, Shimamoto K. Roles of mito-

- chondrial ATP-sensitive K channels and PKC in anti-infarct tolerance afforded by adenosine A1 receptor activation. *J Am Coll Cardiol* 2000; 35: 238–45.
- 12 Son YK, Park WS, Ko JH, Han J, Kim N, Earm YE. Protein kinase A-dependent activation of inward rectifier potassium channels by adenosine in rabbit coronary smooth muscle cells. *Biochem Biophys Res Commun* 2005; 337: 1145–52.
 - 13 Krumschnabel G, Biasi C, Wieser W. Action of adenosine on energetics, protein synthesis and K⁺ homeostasis in teleost hepatocytes. *J Exp Biol* 2000; 203: 2657–65.
 - 14 Darlington DN, Gann DS. Adenosine stimulates NA/K ATPase and prolongs survival in hemorrhagic shock. *J Trauma* 2005; 58: 1–6.
 - 15 Gao J, Mathias RT, Cohen IS, Baldo GJ. Isoprenaline, Ca²⁺ and the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. *J Physiol* 1992; 449: 689–704.
 - 16 Gadsby DC, Nakao M. Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol* 1989; 94: 511–37.
 - 17 Deussen A, Stappert M, Schafer S, Kelm M. Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation* 1999; 99: 2041–7.
 - 18 Gorman MW, Wangler RD, Bassingthwaighe JB, Mohrman DE, Wang CY, Sparks HV. Interstitial adenosine concentration during norepinephrine infusion in isolated guinea pig hearts. *Am J Physiol* 1991; 261: H901–9.
 - 19 Cook MA, Karmazyn M. Cardioprotective actions of adenosine and adenosine analogs. *Exs* 1996; 76: 325–44.
 - 20 Thomas GP, Sims SM, Cook MA, Karmazyn M. Hydrogen peroxide-induced stimulation of L-type calcium current in guinea pig ventricular myocytes and its inhibition by adenosine A1 receptor activation. *J Pharmacol Exp Ther* 1998; 286: 1208–14.
 - 21 Parsons M, Young L, Lee JE, Jacobson KA, Liang BT. Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J* 2000; 14: 1423–31.
 - 22 Mackay K, Mochly-Rosen D. Localization, anchoring, and functions of protein kinase C isozymes in the heart. *J Mol Cell Cardiol* 2001; 33: 1301–7.
 - 23 Henry P, Demolombe S, Puceat M, Escande D. Adenosine A1 stimulation activates delta-protein kinase C in rat ventricular myocytes. *Circ Res* 1996; 78: 161–5.
 - 24 Kudo M, Wang Y, Xu M, Ayub A, Ashraf M. Adenosine A1 receptor mediates late preconditioning via activation of PKC-delta signaling pathway. *Am J Physiol Heart Circ Physiol* 2002; 283: H296–301.
 - 25 Brodsky JL. Insulin activation of brain Na⁺-K⁺-ATPase is mediated by alpha 2-form of enzyme. *Am J Physiol* 1990; 258: C812–7.
 - 26 Gertsberg I, Brodsky I, Priel Z, Danilenko M. Na⁺-K⁺-ATPase in frog esophagus mucociliary cell membranes: inhibition by protein kinase C activation. *Am J Physiol* 1997; 273: C1842–8.
 - 27 Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, *et al*. Nomenclature and classification of purinoceptors. *Pharmacol Rev* 1994; 46: 143–56.
 - 28 Headrick JP, Hack B, Ashton KJ. Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am J Physiol Heart Circ Physiol* 2003; 285: H1797–818.
 - 29 Mubagwa K, Flameng W. Adenosine, adenosine receptors and myocardial protection: an updated overview. *Cardiovasc Res* 2001; 52: 25–39.
 - 30 Kilpatrick EL, Narayan P, Mentzer RM Jr, Lasley RD. Cardiac myocyte adenosine A2a receptor activation fails to alter cAMP or contractility: role of receptor localization. *Am J Physiol Heart Circ Physiol* 2002; 282: H1035–40.
 - 31 Gao J, Wymore RS, Wang Y, Gaudette GR, Krukenkamp IB, Cohen IS, *et al*. Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. *J Gen Physiol* 2002; 119: 297–312.
 - 32 Crambert G, Fuzesi M, Garty H, Karlisch S, Geering K. Phospholemman (FX1D1) associates with Na, K-ATPase and regulates its transport properties. *Proc Natl Acad Sci USA* 2002; 99: 11476–81.
 - 33 Gatto C, Barkulis CT, Schneider WR, Holden JP, Arnett KL, Milanick MA. Inhibition of the Na,K-ATPase by the antiarrhythmic drug, Bretylium. *Ann NY Acad Sci* 2003; 986: 620–2.
 - 34 Gray DE, Mihailidou AS, Hansen PS, Buhagiar KA, Bewick NL, Rasmussen HH, *et al*. Amiodarone inhibits the Na⁺-K⁺ pump in rabbit cardiac myocytes after acute and chronic treatment. *J Pharmacol Exp Ther* 1998; 284: 75–82.