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Effects of Cl^- Channel Blockers on the Cardiac ATP-sensitive K^+ Channel

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To explore whether Cl^- channel blockers interact with the ATP-sensitive K^+ (K_{ATP}) channel, I have examined the effect of two common Cl^- channel blockers on the K_{ATP} channel activity in isolated rat ventricular myocytes using patch clamp techniques. In inside-out patches, 4,4'-diisothio-cyanatostilbene-2,2'-disulfonic acid (DIDS) and niflumic acid applied to bath solution inhibited the K_{ATP} channel activity in a concentration-dependent manner with IC_{50} of 0.24 and 927 μM, respectively. The inhibitory action of DIDS was irreversible whereas that of niflumic acid was reversible. Furthermore, DIDS-induced block was not recovered despite exposure to ATP (1 mM). In cell-attached and inside-out patches, DIDS blocked the pinacidil- or 2,4-dinitrophenol (DNP)-induced K_{ATP} channel openings. In contrast, niflumic acid did not block the pinacidil-induced K_{ATP} channel openings in inside-out patches, but inhibited it in cell-attached patches. DIDS and niflumic acid produced additional block in the presence of ATP and did not affect current-voltage relationship and channel kinetics. All these results indicate that DIDS among Cl^- channel blockers specifically blocks the cardiac K_{ATP} channel.

Key Words: DIDS, Niflumic acid, Cl^- channel, K_{ATP} channel, Ventricular myocyte

INTRODUCTION

Since the first description of ATP-sensitive K^+ (K_{ATP}) channels in cardiac myocytes by Noma (1983), K_{ATP} channel has been identified in other tissues including pancreatic β-cell (Cook & Hales, 1984), skeletal muscle cell (Spruce et al, 1987), neuronal cell (Ashcroft & Ashcroft, 1990) and smooth muscle cell (Standen et al, 1989). The K_{ATP} channels play important functional roles in a variety of tissues by coupling cellular metabolism to electrical activity (Ashcroft & Ashcroft, 1990). In the heart, the K_{ATP} channel is activated during various forms of metabolic stress, including ischemia, hypoxia, and inhibition of glycolysis and/or oxidative phosphorylation. The K_{ATP} channel activity is specifically blocked by sulfonylurea derivatives, such as tolbutamide and glibenclamide (Ashcroft, 1988; Hamada et al, 1990) and activated by a number of K^+ channel openers including pinacidil (Arena & Kass, 1989), nicorandil (Hiraoka & Fan, 1989) and KR-30450 (Kwak et al, 1995).

Recently, it has been discovered that the K_{ATP} channels are composed of at least two kinds of proteins: a pore-forming or inwardly rectifying K^+ channel (Kir) subunit and a ATP-binding cassette (ABC) transporter family such as sulfonylurea receptors (SUR) and cystic fibrosis transmembrane conductance regulator (CFTR) (Aguilar-Bryan et al, 1998). CFTR forms a cAMP-dependent protein kinase A (PKA)-regulated Cl^- channel, has a significant sequence homology with SUR and is sensitive to sulfonylureas (Sheppard & Welsh, 1992). More recently, CFTR was shown to confer sensitivity to sulfonylureas on ROMK2 (McNicholas et al, 1996) and on Kir6.1 channel (Ishida-Takahashi et al, 1998). Inversely, I, therefore, hypothesized Cl^- channel blockers may modulate the K_{ATP} channel activity through binding to SUR.

To explore whether Cl^- channel blockers interact

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with the K_{ATP} channel and to characterize the interaction and improve understanding of the pharmacological modulation and functional structure of the K_{ATP} channel, I have examined effects of two common Cl^- channel blockers on the K_{ATP} channel activity in isolated rat ventricular myocytes. In the present study, it was found that Cl^- channel blockers specifically block the cardiac K_{ATP} channel but that the mode of their K_{ATP} channel inhibition is, in some way, similar to that of glibenclamide.

METHODS

Cell isolation

Single ventricular cells from adult Sprague-Dawley rat (250~300 g) were prepared by enzymatic digestion as described previously (Kwak et al, 1995). After opening chest cavities, hearts were excised and immersed in Krebs-Henseleit (KH) buffer solution (pH 7.35). Each heart was retrogradely perfused via the aorta in a Langendorff apparatus, with KH solution for 5 min to clear visible blood. Hearts were then perfused with Ca^{2+}-free KH solution until they stopped beating, and then they were perfused with Ca^{2+}-free KH solution containing 0.075% collagenase (Worthington, New Jersey, USA) for 30 minutes. After enzymatic digestion, ventricular muscle was removed and placed in Ca^{2+}-free KH solution containing 1% bovine serum albumin, cut into small pieces and mechanically dissociated into single cells. All cells used for experiments were rod-shaped with clear striations. KH solution contained (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 HEPES, 25 $NaHCO_3$, 10 pyruvate, 11 dextrose and 1 $CaCl_2$.

4,4'-diisothio-cyanatostilbene-2,2'-disulfonic acid (DIDS) and niflumic acid were purchased from Sigma (St. Louis, MO, USA).

Electrical recording and data acquisition

Gigaseals were formed with Sylgard-coated pipettes (borosilicate, Kimax) with 4~5 megaohm resistance and single-channel currents were recorded using inside-out and cell-attached configurations of patch clamp technique. The channel currents were recorded with an Axopatch 1D patch clamp amplifier (Axon Instruments, CA, USA) and stored on video tapes via a pulse code modulator (SONY, PCM-501ES, Tokyo, Japan) for computer analysis later. Electrical signals were digitized and stored on hard disk (Hyundai, 40486 DX4, Seoul, Korea) at a sampling rate of 330 kHz using an analog-to-digital converter (Axon Instruments, Digidata1200, CA, USA). The pClamp software (Axon Instruments, V5.7.2, CA, USA) was used for data acquisition and analysis. All experiments were carried out at 22±2°C. The standard bath and pipette solutions contained (in mM) 140 KCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2).

Data analysis

Fifty percent threshold method was used to detect events. The open probability (P_o) was calculated using the equation derived by Spruce et al (1985);

$$P_o = \left(\sum_{j=1}^{N} t_j \cdot j\right) / T_d \cdot N$$

where t_j is the time spent at current levels corresponding to j =0,1,2 .....N channels in the open state. T_d is the duration of the recording and N is the number of channels active in the patch. Recordings of 30~60 sec were analyzed for determination of P_o. The channel activity was expressed as $N \cdot P_o$. Data are presented as means±SEM. For statistical analysis, I compared means using Student's t-test, and a P value of less than 0.05 was considered significant.

RESULTS

Effect of DIDS and niflumic acid on K_{ATP} channel activity

To show directly whether Cl^- channel blockers affect the K_{ATP} channel activity, I first studied the effects of DIDS and niflumic acid on K_{ATP} channel activity in inside-out patches. When inside-out patches were formed in ATP-free bath solution, maximal K_{ATP} channel activities were observed (Fig. 1A). DIDS inhibited the current at a concentration starting as low as 0.1 μM whereas niflumic acid started to inhibit the channel activity at a concentration of 10 μM. When increasing the concentrations of DIDS and niflumic acid were added to the bath solution, K_{ATP} channel activity was inhibited in a concentration-dependent manner. However, >20% of the K_{ATP} channel

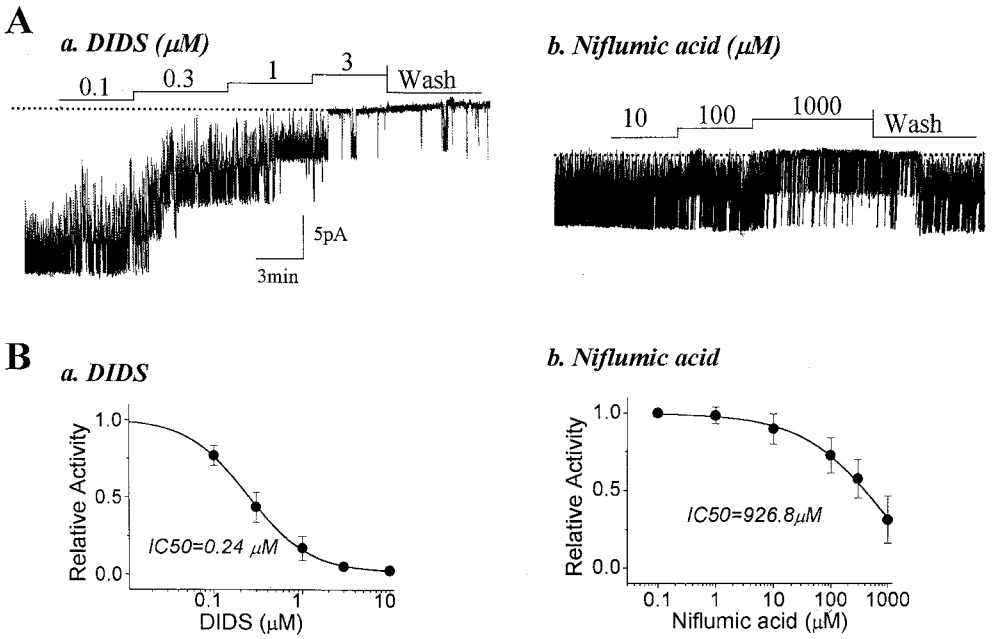

Fig. 1. Effects of intracellular DIDS and niflumic acid on the K_{ATP} channel activity in inside-out patches. All drugs were added to bath solution. Membrane potential was held at −60 mV, and dotted lines denote the resting current level. A: Representative tracings showing inhibitory effect of DIDS (a) and niflumic acid (b) on the K_{ATP} channel activity. Filter for tracing reproduction was set at 300 Hz. B: Relationship between relative channel activity and $[DIDS]_i$ or $[niflumic\ acid]_i$. Each value was obtained from separate patches. Reduction of channel activity by drugs was normalized just to an average of pre-drug and postwashout values of channel activity. Solid lines were fitted to Hill equation as described in the text. Each point with vertical bar denotes the mean with SEM from 5-7 observations.

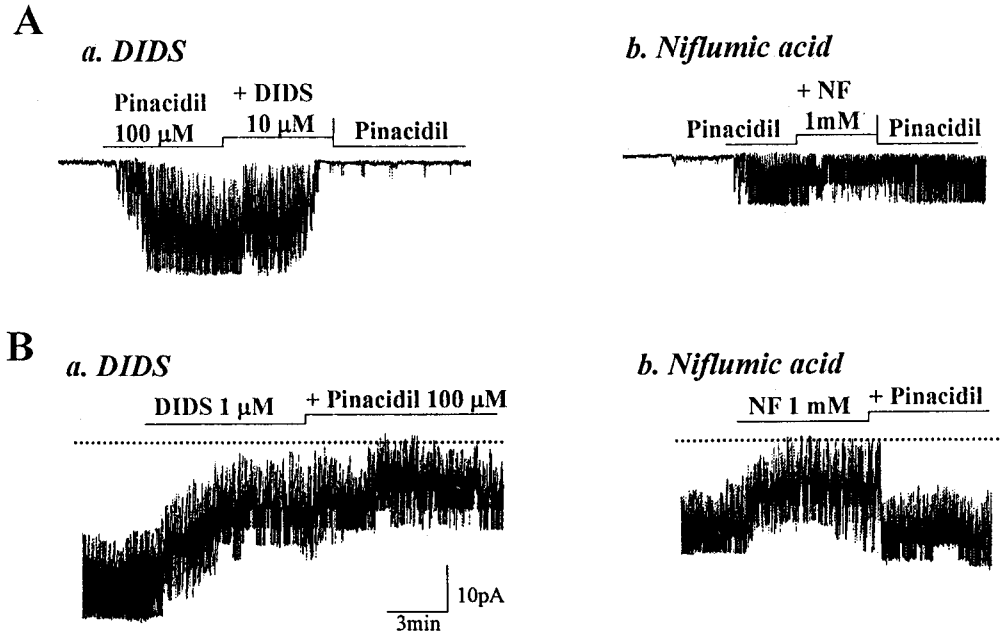

Fig. 2. Effects of DIDS and niflumic acid on the pinacidil (100 μM)-induced K_{ATP} channel activation in cell-attached (A) and inside-out (B) patches. Other information is the same as in Fig. 1.

activity remained even in the presence of 1 mM niflumic acid.

On formation of inside-out patches in ATP-free bath solution, K_{ATP} channel activity was maximally activated but gradually decreased with time. This phenomenon is referred to as "rundown". Therefore, to minimize the time-dependent decrease of the channel activity and to obtain an accurate [Drug]-K_{ATP} channel activity relationship, I determined the effect of one concentration of a drug from each inside-out patch within ~5 min of patch excision. For DIDS, the inhibitory effect was irreversible after washout, whereas washout of niflumic acid resulted in >75% recovery of K_{ATP} channel activity. Plots of relative channel activities as a function of $[DIDS]_i$ or $[niflumic\ acid]_i$ were fitted to the Hill equation using the least-squares method (Fig. 1B); $y=1/\{1+([D]/K_i)^H\}$, where y is the relative $N \cdot P_o$, [D] is the concentration of DIDS or niflumic acid, K_i is the concentration of the drug at which half-maximal inhibition of the channel occurs, and H is the Hill coefficient. For DIDS, the K_i and Hill coefficient were 0.24 μM and 1.25, respectively. For niflumic acid, the K_i and Hill coefficient were 926.8 μM and 0.7, respectively. The inhibitory effect of DIDS was much more potent than that of niflumic acid.

Effects of DIDS and niflumic acid on pinacidil-induced K_{ATP} channel activation

To screen whether Cl^- channel blockers affect the effects of K_{ATP} channel openers, I examined the effects of DIDS and niflumic acid on pinacidil-induced K_{ATP} channel activation in inside-out and cell-attached patches (Fig. 2). Pinacidil is a well-known K_{ATP} channel opener (Edwards & Weston, 1993). In the cell-attached patches (Fig. 2A), no K_{ATP} channel activity was observed in the absence of pinacidil in bath solution. When pinacidil (100 μM) was added to bath solution, it induced the opening of the K_{ATP} channels which had a unitary conductance of about 63 pS at membrane potential of −60 mV. All these channel activities were completely blocked by 10 μM glibenclamide (data not shown). DIDS (10 μM) applied to bath solution completely blocked the K_{ATP} channel-opening effect of pinacidil (100 μM) in cell-attached patches (by 93±5%, n=5), whereas niflumic acid (1 mM) partially blocked it (by 46±5%, n=5). As in inside-out patches, the inhibitory effect of DIDS was irreversible whereas that of niflumic acid was partially reversible after washout. In inside-out patches, pinacidil in the presence of niflumic acid (1 mM) increased the K_{ATP} channel activity by 65±7% (n=6) of the channel activity before pinacidil, whereas pin-

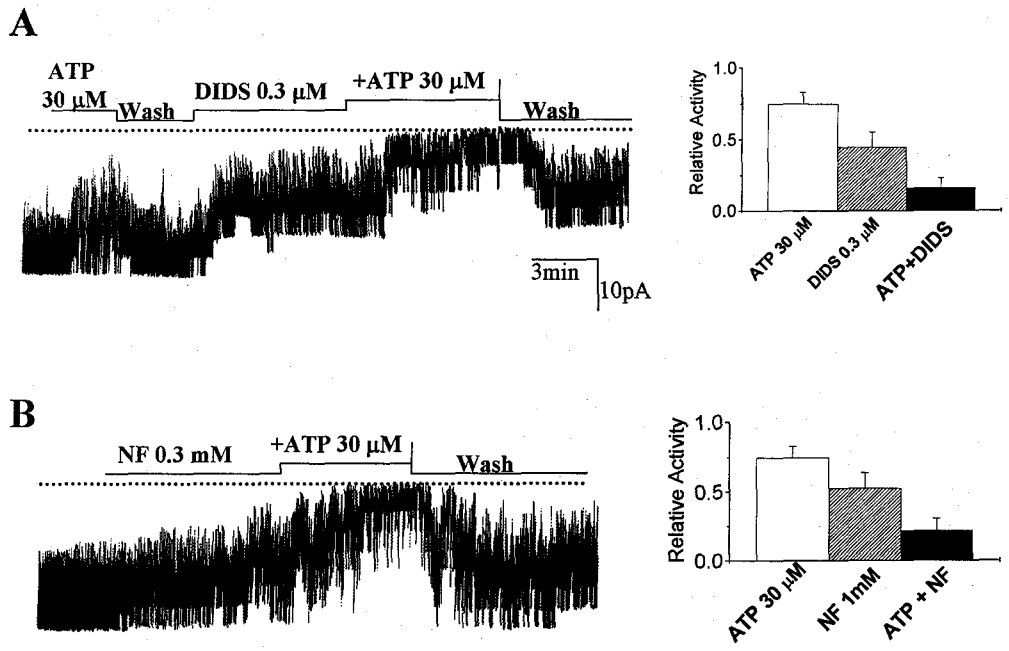

Fig. 3. Effects of intracellular ATP on the K_{ATP} channel activity inhibited by pretreatment with DIDS (0.3 μM; A) and niflumic acid (300 μM; B) in inside-out patches. Other information is the same as in Fig. 1.

acidil in the presence of DIDS (1 μM) did not affect the K_{ATP} channel activity (94±8% of the channel activity before pinacidil, n=7) (Fig. 2B). These results indicate that the binding sites for them might be different.

ATP dependence of DIDS- and niflumic acid-induced inhibition of K_{ATP} channel

To test whether they compete with ATP in inhibiting K_{ATP} channel activity, I examined the inhibitory effects of ATP in the presence of DIDS or niflumic acid (Fig. 3). ATP (30 μM) and DIDS (0.3 μM) blocked the K_{ATP} current by 25±8 and 55±10%, respectively. In the presence of DIDS (0.3 μM), 30 μM ATP produced the additional block of K_{ATP} channel activity (by 84±7%, n=5). These results indicate the independent effects of DIDS or niflumic acid and ATP on K_{ATP} channel inhibition. In cell-attached patches, 2,4-dinitrophenol (100 μM) applied to bath solution induced the opening of the K_{ATP} channels, which was completely blocked by DIDS (100 μM) but partially by niflumic acid (1 mM) (Fig. 4). In addition, when perfused with 2,4-dinitrophenol (100 μM) alone after washout of DIDS or niflumic acid, the channel activity of the DIDS-treated patches was not recovered but that of niflumic acid-treated patches was.

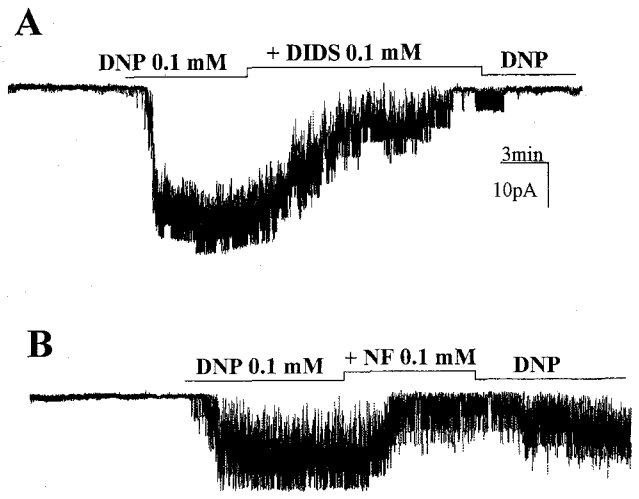

Fig. 4. Effects of DIDS and niflumic acid on the 2,4-dinitrophenol (100 μM)-induced K_{ATP} channel activation in cell-attached patches. Other information is the same as in Fig. 1.

Irreversible K_{ATP} channel block of DIDS

It is well known that exposure to ATP after the K_{ATP} channel rundown could induce the reactivation of the rundown channel. Thus, I tested whether exposure to ATP could reactivate the K_{ATP} channel blocked by DIDS (Fig. 5). 10 μM DIDS completely blocked the K_{ATP} channel, which was not reactivated despite exposure to 1 mM ATP for 5 min (n=5). This result indicates that irreversibility of DIDS-induced inhibition is different from the mechanism of rundown.

Effect of DIDS on the current-voltage relationship and channel kinetics

The current-voltage (I-V) relationship obtained in five patches before and after addition of 1 μM DIDS is shown in Fig. 6. The I-V curves before and after DIDS at negative potentials displayed a linear rela-

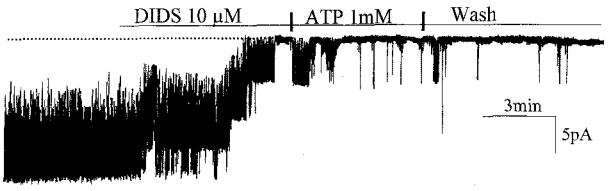

Fig. 5. Irreversibility of DIDS-induced K_{ATP} channel block in inside-out patch. 1 μM ATP was added to bath solution for 5 min after DIDS (10 μM)-induced block occurred. Other information is the same as in Fig. 1.

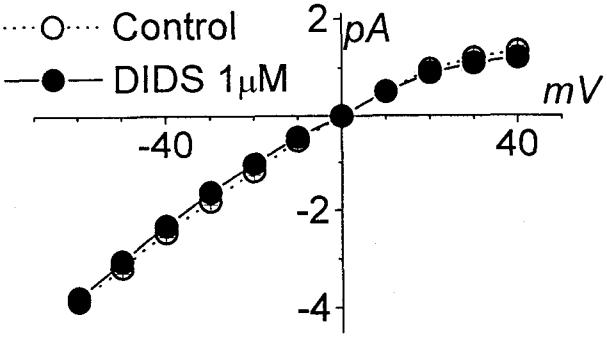

Fig. 6. Effect of DIDS on unitary conductance in inside-out patches. DIDS (1 μM) was applied to the intracellular surface of the patch. Current-voltage (I-V) relationships of K_{ATP} channel were obtained from inside-out patches containing only one active channel. Each point with vertical bar denotes the mean with SEM from 5 observations. Other information is the same as in Fig. 1.

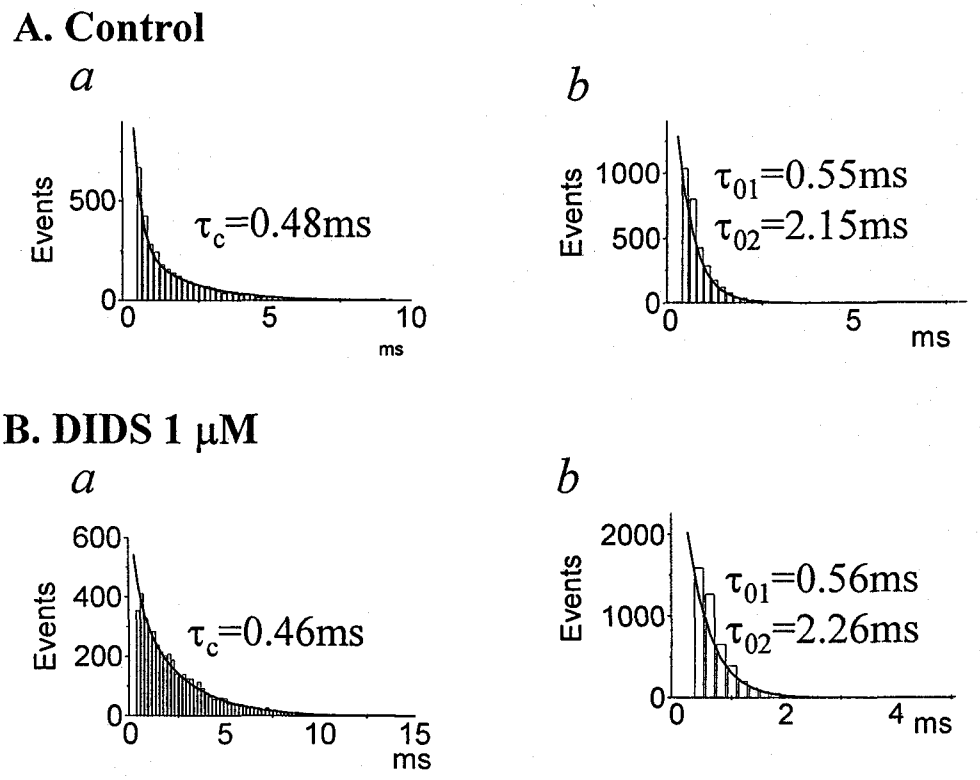

Fig. 7. Effect of DIDS on open and closed time histograms in the absence and presence of DIDS in inside-out patches. DIDS (1 μM) was applied to the intracellular surface of the patch. Data were obtained from inside-out patches containing only one active channel. Curves were fitted with one or two exponential. Other information is the same as in Fig. 1.

tionship with a conductance of 63.5±2.3 and 61.3± 2.5 pS, respectively. The magnitude of inward rectification was not different in both of absence or presence of DIDS.

The open and closed time distributions were analyzed to assess the effect of DIDS on the kinetic properties of the K_{ATP} channel current. In the absence of ATP, the distributions of open and closed time within bursts were measured from patches containing only one active K_{ATP} channel. Open and closed time within bursts and mean burst duration were analyzed at cut-off frequencies of 10 kHz and 0.1 kHz, respectively. Time constants for open and closed time within bursts were not changed by DIDS (1 μM) (Fig. 7), while mean burst duration was slightly shortened by exposure by DIDS (from 53.5±6.1 ms to 44.6±5.8 ms, n=5).

DISCUSSION

In the present study, I have examined the effects of two common Cl^- channel blockers on the K_{ATP} channel. Two structurally distinct compounds were tested: niflumic acid as a kind of fenamates and DIDS as a kind of disulfonic stilbens. These results demonstrate that these Cl^- channel blockers inhibit K_{ATP} channel activity. Surprisingly, this study also show that potencies of these drugs are markedly different.

My results show that DIDS and niflumic acid inhibit the K_{ATP} channel in a different mode. In the present study, DIDS was about 3,800 times more potent in inhibiting K_{ATP} channel activity than niflumic acid (0.24 vs 927 μM). Thus, DIDS-induced K_{ATP} channel block was dependent on its specific chemical structure regardless of its Cl^- channel blocking action. Furthermore, DIDS completely blocked the K_{ATP} channel- opening effect of pinacidil whereas niflumic acid did not affect it. Unlike niflumic acid, DIDS irreversibly blocked the K_{ATP} channel. DIDS-induced block was not recovered even after exposure to ATP (Fig. 5) which is believed to recover the rundown K_{ATP} channels (Ohno-Shosaku et al, 1987). These results indicate that DIDS differently act on

K_{ATP} channels compared with niflumic acid. Especially, the inhibitory action of DIDS showed clear concentration dependency, and occurred at relatively low concentrations (Fig. 1). In other words, IC_{50} for DIDS-induced K_{ATP} block is very low (0.24 μM) compared to the value reported for its blocking action on endothelial Cl^- channel (about 100 μM) (Groschner & Kukovetz, 1992). Thus, the inhibition of K_{ATP} channel by DIDS may be a specific action of disulfonic stilbens. In addition, the difference in reversibility of inhibitory actions between DIDS and niflumic acid may be explained by the presence of isothiocyano structure, as proposed in their inhibitory actions on the anion transporter of erythrocyte membranes (Cabantchik et al, 1978).

DIDS appears to induce K_{ATP} channel inhibition in a manner similar, in some ways, to the blockade of the K_{ATP} channel by a sulfonylurea drug, glibenclamide (Findlay, 1992). The facts that the irreversible block and the slow onset and slow development of inhibitory action by DIDS is similar to that of glibenclamide, could be explained by a "membrane lipid compartment" model, in which the existence of an "intermediate compartment", interpolated between the cytosolic drug source and the site of drug action is hypothesized (Findlay, 1992). In addition, the similarity in channel inhibition by DIDS and sulfonylurea could be expected from their common chemical structures.

DIDS and niflumic acids block Cl^- channels (Groshner & Kukovetz, 1992) and K_{ATP} channel, as shown in this study. Sulfonylureas also inhibit CFTR Cl^- channel as well as K_{ATP} channel (Sheppard & Welsh, 1992). K_{ATP} channel and CFTR Cl^- channel are also similar in their intracellular regulation. Both channels require intracellular ATP to maintain their channel activity (Anderson et al, 1991). These data suggest that K_{ATP} channel and CFTR Cl^- channels have similar pharmacological characteristics. Furthermore, the evidence that CFTR has significant sequence homology with SUR and confers sensitivity to sulfonylureas on ROMK2 (McNicholas et al, 1996) and on Kir6.1 channel (Ishida-Takahashi et al, 1998), support this hypothesis.

Cloning of K_{ATP} channel has revealed that it is an octameric complex of two proteins which assemble with a 4 : 4 stoichiometry (Shyng & Nichols, 1997). The pore-forming subunit, Kir6.2, is a member of the inwardly rectifying K^+ channel family (Inagaki et al, 1995), while the other subunit is an ABC transporter, the sulfonylurea receptors (SUR) (Aguilar-Bryan et al, 1998). The latter endows Kir6.2 with sensitivity to the inhibitory effect of sulfonylurea drugs and to the stimulatory effects of MgADP and K^+ channel openers (Nichols et al, 1996; Trapp et al, 1997; Tucker et al, 1997). The K_{ATP} channels are proposed to form a complex of SUR1 and Kir6.2 in pancreatic β-cells, SUR2A and Kir6.2 in cardiac and skeletal muscles, and SUR2B and Kir6.2 in smooth muscles (Aguilar-Bryan et al, 1998). Considering these reports and my results, the binding site of DIDS on K_{ATP} channel could be dissected. The results showing that DIDS blocked DNP-induced K_{ATP} channel openings (which is due to decrease of intracellular ATP concentration) and induced additional K_{ATP} channel block in the presence of glibenclamide (data not shown), indicate that SUR subunit is not involved in DIDS-induced K_{ATP} block. Furthermore, no competition between DIDS and ATP in inhibiting K_{ATP} channel suggests the binding site for K_{ATP} block of DIDS is not the same to that of ATP. Recently, Drain et al (1998) identified two functional distinct domains of the cytoplasmic C-terminal segment of Kir6.2 subunit, among which one domain is associated with inhibitory ATP binding and another with gate closure. In this study, DIDS blocked the K_{ATP} channel opening induced by low intracellular ATP or pinacidil. Taken together, these data suggest that DIDS may act on Kir6.2 subunit responsible for gate closure, although further studies is needed to prove this hypothesis using Kir6.2 and SUR2A.

Although DIDS and niflumic acid have been shown to be effective blockers of Cl^- channels, their usefulness as pharmacological tools may be greatly limited by their effects on other ion channels such as K_v4 K^+ channels (Wang et al, 1997) and Ca^{2+}-activated K^+ channel (Ottolia & Torro, 1994). Nevertheless, this study characterized the interaction between Cl^- channel blockers and K_{ATP} channel and helps to improve understanding of the pharmacological modulation and functional structure of the K_{ATP} channel. However, further studies are needed to shed light on this study by using cloned channel subunits.

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