ATP-Sensitive Anion Channel from Rat Brain Synaptosomal Membranes Incorporated into Planar Lipid Bilayers

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ABSTRACT An anion channel was incorporated from rat brain synaptic plasma membrane fractions into planar lipid bilayers. The single-channel conductance was found to be 48.5 pS in choline-Cl solution (300 μM cis/100 μM trans). The anion selectivity of the channel was rather low (P Cl/P choline = 1.7). The gating rate of the channel did not change with membrane potential over the range of -50 mV to 50 mV. Several drugs, which are known as inhibitors of anion channels, were found to be efficient inhibitors for the synaptosomal anion channel. 4-Acetoamino-4'-isothiocyanostilbene-2,2'-disulfonic acid, ethacrynic acid, indanylxyacetic acid, and 5-nitro-2-(3-phenylpropylamino) benzoic acid inhibited the channel from the cis side of the membrane, corresponding to the cytoplasmic side of the plasma membrane. We found that the channel is regulated by intracellular ATP at millimolar concentrations. Other nucleotides, ADP and GTP, inhibited the channel as well. Glibenclamide, which is known as an inhibitor of an ATP-regulated potassium channel, inhibited the channel at micromolar concentrations from the trans side of the membrane. It is likely that the synaptosomal anion channel is a member of the ATP-binding cassette superfamily.

INTRODUCTION

A neurotransmitter is released into the synaptic cleft by fusion of the synaptic vesicles, which encapsulate many transmitter molecules, with the plasma membrane at nerve terminals. Recently, many proteins that seem to be related to the fusion process were identified (Matteoli and De Camilli, 1991), although the precise mechanism of the transmitter release is still unknown. On the other hand, the studies of exocytosis in mast cells measuring the capacitance and the conductance of cell membranes have suggested that some membrane proteins form channel-like structures, "fusion pores," at an early step in exocytosis (Zimmerberg et al., 1987; Breckenridge and Almers, 1987). Considering these findings, it is important to characterize ion channels existing in the synaptic plasma membranes and the synaptic vesicle membranes to understand the precise mechanism of nerve transmission. In a previous study, we characterized channels from the synaptic vesicle from rat cerebral cortex by the planar lipid bilayer method (Sato et al., 1992). Recently it was reported that there are a variety of ion channels in synaptic vesicle membranes isolated from the electric organ of Torpedo californica (Kelly and Woodbury, 1996). In this paper we investigate ion channels from synaptic plasma membranes (SPM) by the planar lipid bilayer method and find that there is an anion channel that is regulated by intracellular ATP.

ATP-regulated potassium channels (K_ATP) have been found in many tissues (Edwards and Weston, 1993). It is reported that K_ATP is regulated by intracellular ATP and blocked by sulfonylureas, e.g., glibenclamide and tolbutamide (Edwards and Weston, 1993; Ashcroft and Ashcroft, 1992). Recently the sulfonylurea receptor (SUR) was cloned and its primary structure was determined (Aguilar-Bryan et al., 1995). Interestingly, some inward rectifier potassium channels were coexpressed with SUR to constitute ATP-regulated potassium conductance, suggesting that SUR is a subunit of K_ATP (Inagaki et al., 1995; Sakura et al., 1996). Although there have been detailed studies on K_ATP, only a few studies have been done on ATP-regulated anion channels. Oiki et al. (1994) found that a volume-activated Cl channel is activated by ATP. The human platelet Cl channel (Manning and Williams, 1989) and the Cl channel from the rabbit cortical collecting duct cell (Superdock et al., 1993) are reported to be inhibited by ATP. Schwiebert et al. have reported that the cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-permeable channel and is inhibited by glibenclamide (Schwiebert et al., 1995; Al-Awqati, 1995). Both SUR and CFTR are known as members of the ATP-binding cassette (ABC) superfamily with nucleotide binding folds and multiple transmembrane spanning domains. In this paper we find that the ATP-regulated anion channel from SPM is inhibited by glibenclamide. It is possible that the channel is a member of the ATP-binding cassette superfamily.

MATERIALS AND METHODS

Materials

Adenosine-5'-triphosphate disodium salt (ATP) and adenosine-5'-diphosphate disodium salt (ADP) were purchased from Oriental (Tokyo, Japan), adenosine-5'-monophosphate (AMP) and ethacrynic acid (EA) were from Sigma (St. Louis, MO), guanosine-5'-triphosphate disodium salt (GTP) was from Wako Pure Chemical Industries (Osaka, Japan), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was from Research Biochemi-
Preparation of synaptic plasma membrane vesicles

Synaptic plasma membrane vesicles were prepared according to the method of Jones and Matus (1974), with slight modifications. Fifty milliliters of 10% sucrose buffer (10% sucrose, 4 mM HEPES-NaOH, and 1 mM EGTA, pH 7.3) was added to the forebrains separated from six Sprague Dawley rats, and the tissues were homogenized using a Teflon-glass homogenizer at 900 r.p.m. with 12 strokes. The homogenate was centrifuged at 800 \( \times g \) for 20 min, and the supernatant was centrifuged at 9000 \( \times g \) for a further 20 min. The pellet was suspended in 60 ml of 10% sucrose buffer and then centrifuged at 9000 \( \times g \) for 20 min. For lysis, the pellet was suspended in hypotonic buffer (4 mM HEPES-NaOH and 1 mM EGTA, pH 7.3) and incubated for 30 min at 0°C. This suspension was homogenized at 900 r.p.m. with six strokes and then treated with an ultrasonic sonicator (UC100-2; Olympus, Tokyo, Japan) for 15 s. The lysate was made up to 34% sucrose by the addition of an appropriate volume of 48% sucrose buffer. The 28.5% sucrose buffer was layered on the lysate, and 10% sucrose buffer was layered onto this upper phase to give a sucrose density gradient. These density gradients were centrifuged at 60,000 \( \times g \) for 10 h in a swing-out rotor (RFS25-2; Hitachi, Tokyo, Japan). SPM vesicles were collected at the interphase of the 34% and the 28.5% sucrose layers. The suspension was stored at \(-80°C\).

Electrophysiology and data analysis

Planar lipid bilayers containing 15–20 mg/ml soybean lecithin in \( n \)-decane were painted across a small hole, about 200 \( \mu \)m in diameter, in a polypyrrole cup. In all experiments, the cis chamber is defined as the side to which the SPM vesicles were added, and the opposite side is referred to as the trans chamber. Applied voltages were defined with respect to the trans chamber held at virtual ground. Channel currents were measured under a voltage clamp by a patch-clamp amplifier (CEZ 2200; Nihon Koden, Tokyo, Japan) and digitized and recorded on videotape for later analysis. Data were analyzed with pCLAMP 6.0.2 software (Axon Instruments, Foster City, CA). The cis chamber contained 300 mM choline-Cl, 1 mM EGTA, 2 mM CaCl\(_2\), and 15 mM HEPES (pH 7.3), and the trans chamber contained 100 mM choline-Cl, 1 mM EGTA, 2 mM CaCl\(_2\), and 15 mM HEPES (pH 7.3). The free Ca\(^{2+}\) concentration under these buffer conditions is calculated to be 1.0 mM, by using a program described by Oki (1986) and modified by Kasa. All recordings were carried out under these buffer conditions, unless otherwise noted. Plots of relative open probability, \( P_o/P_o^{(B)} = 0 \), versus drug concentration were fitted to a titration curve of the form \( P_o/P_o^{(B)} = 0 = (1 + ([B]/K_d)^N)^{-1} \), where [B] is the drug concentration, \( N \) is the Hill coefficient, and \( K_d \) represents the dissociation constant of the drug.

RESULTS

Fig. 1 A shows single-channel current fluctuations observed when the synaptosomal anion channel was present in the lipid bilayers. Applied voltages are indicated at the left of each trace. As shown in the figure, the single-channel open probability, \( P_o \), does not vary significantly over the range of \(-45 \text{ mV to } +20 \text{ mV}\). In Fig. 1 B, a plot of single-channel current versus applied voltage shows that the single-channel conductance, \( \gamma \), is 48.5 ± 1.9 pS and that the equilibrium reversal potential under this buffer condition is +6.35 ± 0.9 mV, which corresponds to \( P_{\text{Cl}}/P_{\text{Choline}} = 1.68 \). The current traces in Fig. 1 A suggest that the channel may exist in many distinct states. In these traces, short open and short closed states in bursts and long interburst closed states, sometimes lasting several seconds, are seen. Fig. 1 C shows open and closed time histograms that were constructed from single-channel current traces similar to those shown in Fig. 1 A. The open event histogram was fitted to the sum of a double-exponential function with \( \tau_1 = 1.5 \text{ ms and } \tau_2 = 47 \text{ ms} \). The closed time distribution was fitted with time constants \( \tau_1 = 1.0 \text{ ms and } \tau_2 = 55 \text{ ms} \). These indicate that two open and two closed states were identified when the channel was present in the planar lipid bilayers. Neither the open nor the closed \( \tau \) values changed significantly with change in membrane voltage. The open \( \tau \) values were determined with the same membrane: \( \tau_1 = 1.6 \text{ ms and } \tau_2 = 51 \text{ ms at } -45 \text{ mV} \), and \( \tau_1 = 1.7 \text{ and } \tau_2 = 43 \text{ ms at } -15 \text{ mV} \). The closed time constants were \( \tau_1 = 1.6 \text{ ms and } \tau_2 = 45 \text{ ms at } -45 \text{ mV} \), and \( \tau_1 = 1.8 \text{ ms and } \tau_2 = 50 \text{ ms at } -15 \text{ mV} \).

EA (ethacrynic acid) is known as a diuretic. Landry et al. showed that the anion channels from bovine airway epithelial cells are inhibited by EA (Landry et al., 1987). The current traces in Fig. 2 A are single-channel records taken in the absence or presence of cis EA. The membrane potential was held at \(-30 \text{ mV}\). The addition of EA to the cis chamber results in a decrease in burst duration, whereas \( P_o \) within the burst did not vary significantly. In Fig. 2 B, the relative open probability, \( P_o/P_o^{(B)} = 0 \), is shown as a function of cis EA concentration. Assuming that the drug binds to the channel molecule in a one-to-one manner, the plot of \( P_o/P_o^{(B)} = 0 \) in Fig. 2 B could be fitted with a dissociation constant, \( K_d \), of 14.1 \( \mu \)M. IAA (indanyloxacyclic acid) has a structure analogous to that of EA and was reported to block anion channels from several cells (Landry et al., 1987; Ide et al., 1995). Fig. 2 C illustrates the single-channel behavior observed when the SPM anion channel was present in the planar bilayers in the absence and presence of 10 \( \mu \)M cis IAA. The membrane potential was held at \(-30 \text{ mV} \). \( P_o \) decreased in a manner dependent on cis IAA concentration. \( P_o \) within the burst was decreased by increasing cis IAA concentration, whereas the duration of the burst did not change significantly over the same IAA concentration range. The plot of \( P_o/P_o^{(B)} = 0 \) versus IAA concentration could be fitted with the Hill coefficient \( N = 2.3 \) (Fig. 2 D), suggesting that there are at least two IAA binding sites in the channel molecule. NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid), known as a potent blocker of the anion channel from the kidney (Gekle et al., 1993), was found to inhibit the SPM anion channel. Fig. 3 A illustrates typical single-channel recordings in the presence of NPPB at \(-30 \text{ mV} \). In the case of NPPB, the dose-dependence curve gave \( K_d = 77.7 \mu \text{M} \) and \( N = 1.0 \) (Fig. 3 B). In a previous study, we found that SITS (4-aceatoamino-4'-isothiocyanostilbene-2,2'-disulfonic acid) inhibits the skeletal muscle transverse tubular anion channel (Ide et al., 1995). Fig. 3 C illustrates the single-channel behavior in the presence of 25 \( \mu \text{M} \) cis SITS. The membrane potential was held at \(-30 \text{ mV} \). The closed time within the bursts increased,
resulting in the open probability, $P_o$, decreasing within the bursts. $K_d$ and $N$ were determined to be 24.1 µM and 1.0, respectively.

Fig. 4A shows single-channel recordings in the absence or presence of ATP in the cis chamber. The membrane potential was held at -30 mV. The top trace was recorded in the absence of ATP, and the middle was taken in the presence of 1 mM cis ATP. As shown in these current records, millimolar cis ATP decreased $P_o$. When cis ATP was raised from 0 to 2 mM, $P_o$ decreased from 0.92 to 0.36.

At the bottom it is shown that the effect of ATP was completely removed after perfusion of the cis chamber with ATP-free buffer. ATP in the trans chamber, corresponding to the extracellular space, did not change the gating behavior of the channel. A plot of $P_o/P_o^{[ADP]}$ versus cis ATP concentration was fitted to a single-site titration curve with a $K_d = 1.1$ mM (Fig. 4B). AMP-PNP, a nonhydrolyzable analog of ATP, did not affect the channel gating behavior. Fig. 4C illustrates the effect of cis ADP on the channel. The apparent single-channel conductance was decreased in the
Yuto et al.  Anion Channel from Synaptosomal Membranes 723

FIGURE 2 Effect of anion channel inhibitors on the SPM anion channel. Current records were made at −30 mV. (A) Single-channel current traces taken in the absence (top) and presence (bottom) of 10 μM cis EA. (B) A plot of relative open probability \((P_o/P_{o0})\) versus EA concentration. The data can be fitted to the curve with \(K_d = 14.1 \mu M\). Symbols with error bars represent the mean ± SD (n = 5). (C) Single-channel current traces recorded in the absence and presence of 10 μM cis IAA. (D) A plot of \(P_o/P_{o0}\) versus cis IAA concentration. The fit gives \(K_d = 15.1 \mu M\) and \(N = 2.3\). Symbols with error bars represent the mean ± SD (n = 10).

Presence of millimolar cis ADP, whereas \(P_o\) apparently did not vary. Single-channel conductances were determined to be 43 pS at 2 mM ADP and 33 pS at 5 mM ADP. GTP in the cis chamber also inhibited the channel. The bottom trace in Fig. 4 D is a single-channel record taken in the presence of 1 mM cis GTP and shows that cis GTP produced a fast flickering of the channel. \(P_o\) in this trace was found to be 0.51.

Glibenclamide is known as an inhibitor of ATP-regulated potassium channels (Edwards and Weston, 1993; Ashcroft and Ashcroft, 1992). As shown in Fig. 5 A, the SPM anion channel was inhibited by micromolar concentrations of trans glibenclamide. Glibenclamide in the trans chamber produced a very rapid flickering, and \(P_o\) decreased in a manner dependent on the glibenclamide concentration. The dose dependence curve in Fig. 5 B shows that the \(K_d\) of glibenclamide is 8.3 μM and \(N = 2.0\). Glibenclamide in the cis bilayer solution showed no effect on the channel gating.

DISCUSSION

In this study the synaptic plasma membrane (SPM) was prepared according to the method of Jones and Matus (1974), and the channel within the membrane was studied by reconstitution into artificial planar lipid bilayers by fusion of the vesicles. The planar lipid bilayer system provides an easily studied means of examining the single-channel behavior of the channels within the synaptic plasma membrane, where a patch-clamp pipette is not able to provide easy access.

In previous studies (Nomura et al., 1990; Nomura and Sokabe, 1991), SPM vesicles were found to insert into the planar lipid bilayer in an oriented fashion such that the cis chamber corresponded to the cytoplasmic space and the trans chamber was equivalent to the extracellular space. The Ca\(^{2+}\)-activated K\(^+\) channel, which is known to be present in the synaptic plasma membrane and to be activated by the
cytosolic Ca\textsuperscript{2+} (Nomura et al., 1990), is incorporated into bilayers from the same membrane fraction, and the Ca\textsuperscript{2+} activation site almost exclusively faces the cis side. The anion channel is sometimes incorporated into the bilayers with the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel at the same time, indicating that these channels coexist in one vesicle and fusion of the vesicle results in two different ion-conducting pathways. This evidence shows that the channel investigated in this study originates from the synaptic plasma membrane and that the channel molecule in artificial planar lipid bilayers is oriented as described above.

Nomura and Sokabe reported that there are two types of anion channels, I and II, in a synaptosomal membrane (Nomura and Sokabe, 1991). However, both channels have properties different from those of the channel studied in this paper. Unitary conductances of type I and type II channels were found to be 93 pS and 120 pS in BaCl\textsubscript{2} buffers. These values are much larger than that of the anion channel determined here. The gating of the type I channel showed strong voltage dependency, whereas the channel in this paper is not dependent on membrane potential. Furthermore, the channel investigated in this paper shows low anion/cation selectivity, e.g., \( P_{Cl}/P_{Choline} = 1.68 \), and \( P_{Cl}/P_{Na} = 1.73 \) (data not shown), whereas both type I and type II channels are much more permeable to anions than to cations (\( P_{Cl}/P_{Na} = 4.3 \) and \( P_{Cl}/P_{Na} = 20 \), respectively). These results suggest that both of the channels characterized by Nomura and Sokabe are likely to be distinct from the channel studied here.

Because Ca\textsuperscript{2+} entry into the synaptic terminal triggers transmitter release, it is important to determine the effect of Ca\textsuperscript{2+} on the channel. All current traces shown in the Results section are recorded in the presence of 1 mM cis and trans Ca\textsuperscript{2+}. However, the channel was also active in the absence of Ca\textsuperscript{2+}. To investigate the effect of Ca\textsuperscript{2+} on the channel, single-channel recordings were carried out in the solutions buffered by 1 mM EGTA at various free Ca\textsuperscript{2+} concentrations. Consequently, we have found that the channel is not affected by either cis or trans Ca\textsuperscript{2+}. When cis or trans Ca\textsuperscript{2+} was raised from nanomolar to millimolar concentrations, no
observable change in the gating was seen over the entire Ca\(^{2+}\) concentration range.

We investigated the effects of drugs that are known as anion channel blockers on the SPM anion channel. Both EA and IAA were found to inhibit the channel at tens of mM concentration. These structures are analogous; however, the effects of these two drugs on the channel gating appeared to be different (Fig. 2, A and C). Hill coefficients were determined to be 1.1 for EA and 2.3 for IAA. These findings suggest that there are two binding sites for IAA, whereas
there is only one site for EA. NPPB is known as a potent blocker of anion channels. Gekle et al. found that the Cl conductance of the Madin-Darby kidney cell was inhibited by NPPB with an IC50 = 600 nM (Gekle et al., 1993). NPPB inhibited the SPM anion channel with a Kd of 77.7 μM. In the previous study we found that SITS inhibits the anion channel from skeletal muscle transverse tubular membranes (Ide et al., 1995). We found that the SPM anion channel was inhibited by intracellular SITS. With 25 μM SITS in the cis chamber, the closed lifetimes were increased, resulting in Po within the burst decreasing.

To consider the physiological role of the channel, a comparison with the ATP-regulated K+ channel, KATP, may be helpful. KATP, which is inactivated by intracellular ATP, was found in cardiac muscle, pancreatic b cells, skeletal muscle, smooth muscle, pituitary tissue, and the brain (Edwards and Weston, 1993). KATP is thought to be silent at normal intracellular ATP concentrations. However, it is activated when the intracellular ATP concentration is decreased by abnormal signals, such as ischemia. The resulting efflux sets the cell resting membrane potential or suppresses the excitation of the cell. The SPM anion channel can possibly play a role in stabilizing the membrane potential in a manner similar to that of KATP. We found that P0 of the SPM anion channel decreases in a manner dependent on cis ATP concentration. As shown in Fig. 4 B, the open probability of the channel is about 20% at normal physiological ATP concentrations. When intracellular ATP concentration is decreased, the channel is expected to be activated. The resulting influx of Cl− should be followed by polarization of the plasma membrane of the presynaptic terminal, that is to say, the suppression of transmitter release. However, considering that the anion/cation selectivity of the SPM anion channel is rather low, it is impossible to bring the membrane to a resting state by activating only this channel. To elucidate the function of the channel, the distribution of the channel molecules in the synaptic plasma membrane and the exact gradient of ion concentrations across the membrane should be determined.

The effect of ATP on the SPM anion channel was completely reversed by perfusion of the chamber with ATP-free buffer. This suggests that ATP regulates the channel activity by direct binding to the channel molecule and not by phosphorylating the channel, although a nonhydrolyzable analog, AMP-PNP, showed no effect on the gating. Because GTP inhibited the channel in a manner similar to that of ATP, it is likely that the phosphate group of these nucleotides plays an important role in the binding to the channel molecule. We found that intracellular ADP inhibits the channel in a different manner from ATP. ADP appeared to decrease the single-channel conductance without affecting the open probability. However, it is possible that, in the presence of ADP, the change in conductance was so rapid that individual openings were blurred out by the limited band width of the amplifier, and the apparent conductance was lowered. Although it is unknown whether the difference between the effect of ATP and that of ADP is mere appearance, it is interesting that ATP and ADP regulate the channel in a different manner. If the channel is regulated simultaneously by ATP and ADP, or by the ratio of ATP to ADP, the channel should influence the ATP metabolic pathway.

The ATP-regulated K+ channels are known as the target for the sulfonylureas, oral hypoglycemic agents widely used in the treatment of non-insulin-dependent diabetes mellitus to stimulate insulin release from pancreatic islet b cells. The mechanism of stimulation is through inhibition of KATP (Edwards and Weston, 1993; Ashcroft and Ashcroft, 1992). The decrease in potassium outflow causes depolarization of the membrane and triggers the voltage-activated Ca2+ channels. The resulting Ca2+ influx activates exocytosis. The sulfonylurea receptor, SUR, is a member of the ATP-binding cassette (ABC) superfamily with multiple membrane-
spanning domains and nucleotide-binding folds (Aguilar-Bryan et al., 1995). Recently it was reported that the inward rectifier potassium channel was coexpressed with SUR to reconstitute ATP-regulated potassium conductance, indicating that the SUR is a subunit of $K_{ATP}$ and regulates the ATP sensitivity of the channel (Inagaki et al., 1995; Sakura et al., 1996). The cystic fibrosis transmembrane conductance regulator, CFTR, which is another member of the ABC superfamily, is an anion-selective channel that is activated by cAMP (Cliff et al., 1992). Schwiebert et al. found that the CFTR is inhibited by glibenclamide and that it is an ATP-permeable channel (Schwiebert et al., 1995). As shown in Fig. 5, the SPM anion channel was inhibited by micromolar trans glibenclamide. Effects of ATP and glibenclamide on the SPM channel suggest that the channel has a structure similar to that of CFTR; namely, it is possible that the SPM anion channel is a member of the ABC superfamily.

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