Interaction of Vanadate with the Cloned Beta Cell K$_{ATP}$ Channel*

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Vanadate is used as a tool to trap magnesium nucleotides in the catalytic site of ATPases. However, it has also been reported to activate ATP-sensitive potassium (K$_{ATP}$) channels in the absence of nucleotides. K$_{ATP}$ channels comprise Kir6.2 and sulfonylurea receptor subunits (SUR1 in pancreatic beta cells, SUR2A in cardiac and skeletal muscle, and SUR2B in smooth muscle). We explored the effect of vanadate (2 mM), in the absence and presence of magnesium nucleotides, on different types of cloned K$_{ATP}$ channels expressed in oocytes. Currents were recorded from inside-out patches. Vanadate inhibited Kir6.2/SUR1 currents by ∼50% but rapidly activated Kir6.2/SUR2A (4-fold) and Kir6.2/SUR2B (2-fold) currents. Mutations in SUR that abolish channel activation by magnesium nucleotides did not prevent the effects of vanadate. Studies with chimeric SUR indicate that the first six transmembrane domains account for the difference in both the kinetics and the vanadate response of Kir6.2/SUR1 and Kir6.2/SUR2A. Boiling the vanadate solution, which removes the decavanadate polymers, largely abolished both stimulatory and inhibitory actions of vanadate. Our results demonstrate that decavanadate modulates K$_{ATP}$ channel activity via the SUR subunit, that this modulation varies with the type of SUR, that it differs from that produced by magnesium nucleotides, and that it involves transmembrane domains 1–6 of SUR.

ATP-sensitive potassium (K$_{ATP}$) channels are found in a variety of tissues where they couple changes in cellular metabolism to electrical activity and potassium fluxes (1–3). Molecular cloning of these channels has revealed that they consist of two distinct types of subunit (a pore-forming subunit (Kir6.2) and a sulfonylurea receptor subunit (SUR1 in pancreatic beta cells, SUR2A in cardiac and skeletal muscle, and SUR2B in smooth muscle)) that associate in a 4:4 stoichiometry to form an octameric K$_{ATP}$ channel. (4–12). The sulfonylurea receptor subunit belongs to the ATP-binding cassette (ABC) transporter family and is characterized by multiple transmembrane domains and two large cytosolic loops that contain consensus sequences for nucleotide binding and hydrolysis (4, 13). Like the nucleotide-binding domains (NBD) of other ABC transporters, those of the sulfonylurea receptor each possess a Walker A and a Walker B motif. Adenine nucleotides exert both stimulatory and inhibitory effects on K$_{ATP}$ channel activity that are mediated by separate subunits. Thus, interaction of ATP with Kir6.2 causes the channel to close, whereas interaction of MgADP or MgATP with the NBDs of SUR enhances K$_{ATP}$ channel activity (14–17). A number of studies have directly demonstrated ATP binding and hydrolysis by the nucleotide-binding domains of several members of the ABC transporter family, including P-glycoprotein (MDR; Ref. 18), the multidrug resistance-like protein (MRP; Ref. 19), the cystic fibrosis gene product (CFTR; Ref. 20), and the bacterial maltose transporter (21).

Vanadate is routinely used as a tool to trap magnesium nucleotides in the catalytic site of ATPases. ADP is trapped by vanadate both as a result of ATP hydrolysis and also when it is added directly to the solution (22). This is likely to be because orthovanadate acts as an analogue of phosphate. Among eukaryotic ABC transporters, vanadate trapping has been demonstrated for both MDR (18) and MRP (19). Photoaffinity labeling experiments with 8-azido-$^{32}$P]ATP have revealed that ATP binds with high affinity to NBD1 of SUR1 and that MgADP binds to NBD2 (23). Unlike other ABC transporters, however, vanadate binding to SUR1 is not enhanced by vanadate (23).

The effects of vanadate on K$_{ATP}$ channel currents are variable. It has been reported to enhance the activity of K$_{ATP}$ channels in skeletal muscle (24) and in ventricular myocytes (25), but it had no effect on the cloned K$_{ATP}$ channel Kir6.2/SUR1 in the presence of magnesium nucleotides (16). One explanation for these disparate findings is that different types of K$_{ATP}$ channel exhibit different sensitivities to vanadate. Another reason may be that vanadate interacts with a third protein, not present in the heterologous expression system, to modulate K$_{ATP}$ channel activity. A third possibility is that, because vanadate exists in solution in a number of different polymeric forms (e.g. orthovanadate and decavanadate), the concentration of the different vanadate complexes might vary in the experimental solutions used by different investigators.

In this paper, we examine the effects of orthovanadate and decavanadate on the activity of cloned K$_{ATP}$ channels containing different types of SUR subunit, heterologously expressed in Xenopus oocytes. We show that the major effects of vanadate on K$_{ATP}$ channel activity are mediated via the sulfonylurea receptor subunit, with different SURs producing different modulatory effects. We further demonstrate that the interaction of vanadate with SUR is not mediated via the nucleotide-binding domains and that the first six transmembrane domains are required for activation of SUR2 by vanadate. This region also determines the different kinetics of Kir6.2/SUR1 and Kir6.2/SUR2 channels.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—Mouse Kir6.2 (GenBank™ accession number D50581, Refs. 5 and 11), rat SUR1 (GenBank™ accession number L40624, Ref. 4; provided by Dr. G. Bell, University of Chicago), rat SUR2A (GenBank™ accession number D83598, Ref. 6; provided by

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The abbreviations used are: K$_{ATP}$, ATP-sensitive potassium channel; SUR, sulfonylurea receptor; ABC, ATP-binding cassette; NBD, nucleotide-binding domain; MDR, multidrug resistance protein; TM, transmembrane domain.

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Dr. S. Seino, Chiba University School of Medicine, and rat SUR2B (GenBank\textsuperscript{TM} accession number AF199682, Ref. 8) were subcloned into the pBF expression vector that provides the 5'- and 3'-untranslated regions of the Xenopus \(\beta\) -globin gene. A 36-amino acid C-terminal deletion of mouse Kir6.2 (GenBank\textsuperscript{TM} accession number D25394, Ref. 8) was made by introduction of a stop codon at the single stop residue (171). Site-directed mutagenesis of SUR was carried out by subcloning the appropriate fragments into the pALTER vector (Promega). We use the abbreviations SUR1-KA/KM and SUR2A-KA/KA to refer to mutation of the Walker A lysines in NBD1 and NBD2 of SUR, to alanine (A) or methionine (M). In SUR1, this corresponds to K719A and K1385M and in SUR2A to K707A and K1354M. A single mutation in NBD1 of SUR2B was also introduced (SUR2B-KA). SUR chimeras containing different segments of SUR1 were constructed by standard molecular biology techniques. The chimeras were composed of the following segments, where the numbers refer to the sequences of SUR1 (GenBank\textsuperscript{TM} accession number L40624) or SUR2A (GenBank\textsuperscript{TM} accession number D83598) as appropriate: SUR21-a, (1–416, SUR2A)-(424–700, SUR1); SUR21-b, (1–1248, SUR2A)-(1285–1581, SUR1); SUR21-c, (1–308–1545, SUR2A); SUR21-x, (1–1013, SUR2A)-(1035–1277, SUR1)-(1–1242–1545, SUR2A). Synthesis of capped mRNA was carried out using the mMessage mMachine in vitro transcription kit (Ambion). Amino acids are indicated by the single-letter code.

**Oocyte Handling**—Female Xenopus laevis were anesthetized with MS222 (2 g/liter added to the water). One or two ovaries were removed via a minilaparotomy, the incision was sutured, and the animal was allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation, and the animal was killed by decapitation while under anesthesia. Immature stage V-VI Xenopus oocytes were incubated for 60 min with 0.1 mg/ml collagenase (type V, Sigma) and then manually defolliculated. In most experiments, oocytes were injected with 2 ng of mRNA encoding SUR (either the wild-type or mutant form of SUR1, SUR2A, or SUR2B) and ~0.04 ng of Kir6.2 mRNA. In some experiments, oocytes were injected with 2 ng of mRNA encoding Kir6.2AC36. The final injection volume was ~50 nl/oocyte. Isolated oocytes were maintained in Barth’s solution, and currents were studied 1–4 days after injection (26).

**Electrophysiology**—Patch pipettes were pulled from thick-walled glass and had resistances of 250–500 kilo-ohms when filled with pipette solution. Macroscopic currents were recorded from giant inside-out patches using an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany) at 20–24 °C (26). The holding potential was 0 mV, and currents were evoked by repetitive 3-s voltage ramps from −110 to +100 mV. Currents were filtered at 0.2 kHz, digitized at 0.5 kHz using a Digidata 1200 Interface, and analyzed using pClamp software (Axon Instruments, Burlingame, CA). The data were also stored on video tape (210 kHz), and records for display in the figures were resampled at 20 Hz. Single-channel currents were recorded at ~60 mV, filtered at 5 kHz, and sampled at 20 kHz.

The pipette solution contained (in mM): KCl, 140; MgCl\(_2\), 1.2; CaCl\(_2\), 2.6; and HEPES, 10 (pH 7.4 with KOH). The internal (bath) solution contained (in mM): KCl, 155; MgCl\(_2\), 5; KOH, 5; EGTA, 1; HEPES, 10 (pH 7.2 with KOH); and nucleotides as indicated. Sodium vanadate (Na\(_3\)VO\(_4\)) was added to the solution at a concentration of 2 mM, and the pH was adjusted to 7.2 with KOH. At concentrations higher than ~100 \(\mu\)M, vanadate exists in solution as a number of polymeric forms (27). At room temperature and pH 7.2, the vanadate monomer (orthovanadate) coexists in equilibrium with small amounts of divanadate and tetravanadate (28). A small amount of decavanadate is also generated immediately after pH readjustment, and its presence is indicated by the characteristic yellow color of the solution (27). Because decavanadate is not stable at pH 7.2 and slowly decomposes with time (27), solutions were made up just before starting the experiment and were only used for 1–2 h. To prevent chelation of vanadate species by EGTA, we used a low concentration of the chelator (1 mM) and increased the Mg\(^{2+}\) concentration to 5 mM. Boiling the vanadate solution for ~30 min permanently removes decavanadate, which can be visualized by a change of color from yellow (decavanadate) to colorless (27). Boiled solutions contain mostly orthovanadate, although bivanadate and tetravanadate may also be present (28, 29). In this paper, we refer to the unboiled solution as “vanadate solution” and the boiled solution as “decavanadate-free vanadate solution.” Solutions containing vanadate were made up fresh each day, and the pH was readjusted after addition of Na\(_2\)VO\(_4\) with HCl. For decavanadate-free solutions, ADP was added after boiling. Rapid exchange of solutions was achieved using a rapid solution changer (RSC-2000, Biologic, Claireix, France).

**Data Analysis**—The slope conductance was measured by fitting a straight line to the current-voltage relation between ~100 mV and ~20 mV; the average of five consecutive ramps was calculated in each solution. Currents were corrected by subtraction of the background current measured in water-injected oocytes (~5 pA at −100 mV). Conductance was expressed as a fraction of the mean of that obtained in control solution before and after ATP application. The data are given as the mean ± S.E. The symbols in the figures indicate the mean, and the vertical bars represent one S.E. (where this is larger than the symbol). Statistical significance was tested by Student’s \(t\) test.

The concentration dependence of the activatory and inhibitory effects of vanadate on channel activity were fitted according to Equations 1 and 2,

\[
G/G_0 = \frac{1 + G_{\text{max}}[V]/EC_{50}}{1 + [V]/EC_{50}} \quad (\text{Eq. 1})
\]

\[
G/G_0 = (1 + G_{\text{max}}[V]/EC_{50})/(1 + [V]/EC_{50}) \quad (\text{Eq. 2})
\]

where \(G\) and \(G_0\) are the macroscopic conductances in the presence and absence of decavanadate, respectively, \([V]\) is the total vanadate concentration, \(EC_{50}\) is the concentration of vanadate at which the effect is half-maximal, \(h\) is the Hill coefficient, \(G_{\text{max}}\) is the maximal fractional activation of vanadate, and \(L\) is the fraction of remaining current unblocked by vanadate. For analysis of the inhibitory effect of decavanadate, all values were corrected for the small inhibitory effect of the decavanadate-free solution on Kir6.2/SUR1 currents (4%; see Table I).

**RESULTS**

**Effects of Vanadate on Channel Activity**—Fig. 1. A and B, shows that intracellular application of 2 mM sodium vanadate (Na\(_3\)VO\(_4\)) produced a marked stimulation of Kir6.2/SUR2A currents and a somewhat smaller increase in Kir6.2/SUR2B currents; the mean increase in the conductance was 454 ± 127% (\(n = 7\)) for Kir6.2/SUR2A and 174 ± 24% (\(n = 8\)) for Kir6.2/SUR2B (Table I). In contrast, vanadate blocked Kir6.2/SUR1 currents (Fig. 1C). The extent of this block varied between oocyte preparations, with a mean inhibition of 55 ± 9% (\(n = 18\)). The block was slow and only partially reversible. In some experiments (Fig. 1C, bottom), removal of vanadate caused an initial decrease and then an increase in current, suggesting that the ion might exert more than one effect on Kir6.2/SUR1.

When Kir6.2AC36 was expressed in the absence of SUR1, vanadate produced only a very small (~5%) inhibition of the current (Fig. 1D and Table I). This result indicates that the SUR subunit, rather than Kir6.2, is primarily responsible for the effects of vanadate on the wild-type K\(_{\text{ATP}}\) channel, a view that is supported by the disparate actions of vanadate on K\(_{\text{ATP}}\) channels containing different types of SUR subunit.

**E affects of Decavanadate-free Solution on Channel Activity**—We next explored whether the effects of vanadate were mediated by the decavanadate species. To address this question, we boiled the vanadate solution to remove decavanadate. As shown in Table I, the decavanadate-free vanadate solution was without marked effect on the amplitude of Kir6.2AC36, Kir6.2/SUR1, Kir6.2/SUR2A, or Kir6.2/SUR2B currents. The mean conductance, expressed as a percentage of its value in control solution, was 93%, 96%, 93%, and 95% for Kir6.2AC36, Kir6.2/SUR1, Kir6.2/SUR2A, and Kir6.2/SUR2B currents, respectively (Table I). These results indicate that the effects of vanadate solutions on channels containing SUR subunits are not mediated by the orthovanadate complex but rather by the decavanadate form. The small decrease in current observed for all types of K\(_{\text{ATP}}\) channel is similar to that observed for the effect of vanadate solution on Kir6.2AC36 currents and argues that this effect is mediated via interaction with the Kir6.2 subunit and by vanadate species other than decavanadate.

**Concentration Dependence of Vanadate-induced Effects**—To compare the sensitivity of cloned and native K\(_{\text{ATP}}\) channels, we measured the concentration-response curves for the effects of vanadate on Kir6.2/SUR2A and Kir6.2/SUR1 currents (Fig. 2). The EC\(_{50}\) for vanadate activation of Kir6.2/SUR2A currents was 0.59 ± 0.03 mM, with a Hill coefficient of 1.8 ± 0.2 and
maximum activation of 3.25 ± 0.06 (n = 5). The EC_{50} was slightly lower than the value (1.5 mM) reported by Neumcke and Weik (24) for native cardiac K_{ATP} channels but similar to that obtained by Nakashima et al. (25) in the presence of 300 μM ATP (0.5 mM). The inhibitory effect of vanadate on Kir6.2/SUR1 had an EC_{50} of 0.28 ± 0.02 mM and a Hill coefficient of 2.9 ± 0.5 (n = 5). Approximately 30% of the current was unaffected by vanadate. That the Hill coefficients for both activation and inhibition of K_{ATP} currents were larger than one suggests that the cooperative action of more than one vanadate molecule is involved in these effects. The fact that vanadate was without effect at a concentration of 0.1 mM supports the idea that the effects are mediated by vanadate polymers, which are only generated in solutions containing >0.1 mM vanadate (27).

Effects of Decavanadate-free Solutions on the Response to MgADP—Vanadate-trapping effects are generally assigned to the orthovanadate species (30, 31). Magnesium nucleotides enhance K_{ATP} channel activity, with the most dramatic effects being caused by MgADP. Therefore, we examined the effect of decavanadate-free solution on the ability of MgADP to enhance the activity of the K_{ATP} channel. If orthovanadate causes nucleotide trapping, we would expect it to influence the stimulatory effect of MgADP. In control solution, 3 μM and 100 μM MgADP increased Kir6.2/SUR1 currents by ~28% and ~70%, respectively (Fig. 3 and Table II). There was no change in the effect of MgADP when the nucleotide was applied in the presence of decavanadate-free vanadate solution. Shyng et al. (16) have also reported that vanadate has no effect on the potentiating action of MgADP on Kir6.2/SUR1 currents.

Effects of Mutations in SUR on Decavanadate Action—Our results indicate that the effects of decavanadate are mediated by the sulfonylurea receptor and not by the Kir6.2 subunit. The stimulatory effects of MgADP and of potassium-channel openers such as diazoxide are impaired by mutations in the NBDs of SUR (14–16). To determine whether this is also the case for decavanadate, we examined the effects of mutations that are known to disrupt the activation of Kir6.2/SUR1 currents by both MgADP and diazoxide (14, 16); thus we changed one or both lysines in the Walker motifs of NBD1 and NBD2 to alanine or methionine. The effect of vanadate solution on K_{ATP} channels containing SUR1, SUR2A, or SUR2B carrying these mutations was unchanged, indicating that the mechanism of vanadate activation is distinct from that of MgADP (Table I).

Effects of Decavanadate on SUR Chimeras—To identify regions of SUR2 involved in the activation of the K_{ATP} channel by decavanadate we constructed a range of chimeras between SUR1 and SUR2A, based on the sequence of SUR2A, and coexpressed these chimeric SURs with Kir6.2. The results are summarized in Fig. 4.

SUR2A containing the second nucleotide-binding domain of SUR1 (chimera SUR21-u) or possessing transmembrane do-
mains (TMs) 8–11 of SUR1 (chimera SUR21\textsuperscript{-v}) behaved like SUR2A. There was also no effect of transferring TMs 12–17 (chimera SUR21\textsuperscript{-x}), which contains the tolbutamide binding site (32). When the first six TMs of SUR2A were replaced with those of SUR1 (SUR21\textsuperscript{-w}), however, the current was not activated but was instead inhibited by vanadate solution in a manner similar to Kir6.2/SUR1 (see Fig. 6B). This suggests that the first six TMs of SUR are responsible for the different decavanadate sensitivities of SUR1 and SUR2A. As previously reported (33), exchange of this region also influenced the gating properties of the KATP channel. Like Kir6.2/SUR1, channels comprising Kir6.2/SUR21\textsuperscript{-w} exhibited shorter bursts and shorter long closed states than Kir6.2/SUR2A (Fig. 5).

**DISCUSSION**

The data presented in this paper clearly demonstrate that decavanadate interacts with the sulfonylurea receptor subunit of the K\textsubscript{ATP} channel and that the effect of this interaction varies with the type of SUR subunit, being largely inhibitory for...
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channels containing SUR1 and stimulatory for channels containing SUR2A or SUR2B. Thus, decavanadate can be used as a tool to determine which type of SUR subunits are present in \( K_{ATP} \) channels from various tissues. The different effects reported previously for vanadate action on native \( K_{ATP} \) channels in skeletal muscle (24) and on cloned Kir6.2/SUR1 channels (16) therefore result from differences in the SUR subunit of these channels (skeletal muscle is believed to contain SUR2A; see Ref. 6) rather than from differences in native and cloned channels per se. The fact that Shyng et al. (16) observed results similar to those we report when Kir6.2/SUR1 was expressed in mammalian cells further demonstrates that the action of vanadate is not influenced by the choice of expression system.

Our results suggest that the effects of vanadate are mediated by the decavanadate polymer and that orthovanadate is without marked effect either in the absence or presence of magnesium nucleotides, because the effects of vanadate on both SUR1 and SUR2A are abolished by boiling the solution, which destroys the decavanadate form. Furthermore, vanadate was without a substantial effect at a concentration of 0.1 mm, where vanadate polymers are virtually absent. This is in agreement with experiments demonstrating that orthovanadate does not influence MgATP binding to the sulfonyleurea receptor SUR1 (23). In this respect, SUR1 differs from other members of the ABC transporter family, such as MDR and P-glycoprotein, where nucleotide trapping by orthovanadate has been demonstrated in binding studies (18, 19, 31).

Two pieces of evidence demonstrate that decavanadate interacts with the SUR, rather than the Kir6.2, subunit of the \( K_{ATP} \) channel. First, decavanadate is largely ineffective when Kir6.2ΔC36 is expressed in the absence of SUR. Second, the effect of vanadate is influenced by the type of SUR subunit that the \( K_{ATP} \) channel contains. In contrast to SUR2A, SUR1 is inhibited by decavanadate. It is possible that decavanadate also exerts an inhibitory effect on SUR2A but that this is masked by the presence of an additional stimulatory action. The fact that decavanadate blocks a chimera consisting of SUR2A with the first six TMs of SUR1 might be consistent with this view. Conversely, the variable extent of inhibition of SUR1 produced by vanadate might suggest that the ion also exerts a small stimulatory effect on SUR1.

Previous studies have suggested that decavanadate activates cardiac \( K_{ATP} \) channels in a way similar to MgADP (25). In particular, both compounds enhance the channel activity without affecting the single-channel conductance, both are able to reactivate rundown channels, and both require Mg\(^{2+}\) for their action. Our results indicate, however, that the mechanism of action of vanadate and MgADP is not identical, because decavanadate is able to stimulate Kir6.2/SUR2A and Kir6.2/SUR2B channels when MgADP activation has been abolished by mutation of the nucleotide-binding domains of SUR. Instead, our studies suggest that the first six transmembrane domains and/or the cytosolic loop between TMs 5 and 6 play a critical role in channel activation either because this region contains the binding site for decavanadate or because it is involved in transducing binding into channel activation. It is noteworthy that the first six TM of SUR are involved not only in the action of vanadate but also mediate the effect of SUR on channel gating. Thus this region may be important for transduction of vanadate binding to SUR into changes in channel gating.

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