Activation of the Calcium Release Channel (Ryanodine Receptor) by Heparin and Other Polyanions is Calcium Dependent

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Heparin has been used as a potent competitive inhibitor of inositol 1,4,5-trisphosphate (IP₃)-binding to IP₃ receptors and to block IP₃-gated calcium channels in bilayer experiments. In contrast to the effect on the IP₃-gated channel, heparin (0.1–1 µg/ml) opened the Ca release channel (ryanodine receptor). Other polyanions such as pentosan polysulfate and polyvinyl sulfate also activated the Ca release channel. The effect of polyanions on the Ca release channel was Ca dependent. Polyanion addition activated the Ca release channel when free Ca was >80 nM, but was ineffective when free Ca was <20 nM. The level of channel activation could be altered by manipulating the free Ca concentration. These results suggest that the polyanions act by increasing the local concentration of Ca near regulatory sites on the channel complex. As most cells have both types of intracellular channels, the opposite effects of the polyanions on the two channel types suggests that addition of polyanions to intact cells may produce multiple effects.

INTRODUCTION

Two types of calcium release channels (Ca-gated Ca channels and inositol 1,4,5-trisphosphate (IP₃)-gated Ca channels) have been identified in endoplasmic/sarcoplasmic reticulum. Both types of Ca channels appear to be present in a number of tissues, though the relative abundance differs. The Ca-gated Ca channel (also known as the ryanodine receptor), e.g., is abundant in sarcoplasmic reticulum of striated muscle and plays an important role in excitation-contraction coupling (Fleischer and Inui, 1989). Some IP₃-gated channel activity has also been detected in striated muscle (Suarez-Isla *et al.*, 1988; Borgatta *et al.*, 1991). Smooth muscle and cerebellum, by contrast, have a higher density of IP₃ receptors than ryanodine receptors (Ross *et al.*, 1989; Chadwick *et al.*, 1990; Ellisman *et al.*, 1990). Consistent with the latter studies, IP₃-gated Ca channels are much

more prevalent in cerebellum and smooth muscle than are Ca-gated Ca channels (Ehrlich and Watras, 1988; Bezprozvanny *et al.*, 1991).

Recently, marked progress has been made in our understanding of these two channels. Both the ryanodine receptor and the IP₃ receptor have been cloned (Furuichi *et al.*, 1989; Mignery *et al.*, 1989; Takeshima *et al.*, 1989; Nikai *et al.*, 1990), and some structural similarities were noted between these channel proteins (Mignery *et al.*, 1989; Nikai *et al.*, 1990). Each channel type exists as a tetramer, containing four receptors with fourfold symmetry (Lai *et al.*, 1988; Wagenknecht *et al.*, 1989; Chadwick *et al.*, 1990; Maeda *et al.*, 1990). Interaction between receptors within a given channel complex has been inferred by the appearance of up to four conductance levels in both channel types (Smith *et al.*, 1988; Watras *et al.*, 1991a). Differences between these two channels have been noted in terms of molecular weight (Supattapone *et al.*, 1988; Chadwick *et al.*, 1990), conductance (Ehrlich and Watras, 1988; Smith *et al.*, 1986), and pharmacology (Ehrlich and Watras, 1988; Palade *et al.*, 1989).

In the present study, we have used heparin and several other polysulfated polyanions to further compare

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these two Ca channels. Heparin is a potent inhibitor of IP₃ binding (Supattapone *et al.*, 1988) and IP₃-induced Ca release (Ghosh *et al.*, 1988; Kobayashi *et al.*, 1988) in a variety of tissues. In contrast, heparin induced Ca release from vesicles made from skeletal muscle sarcoplasmic reticulum (Ritov *et al.*, 1985). To determine the mechanism of the unexpected effect of heparin in skeletal muscle sarcoplasmic reticulum vesicles, we tested the effects of polyanions on Ca-gated Ca channels incorporated into planar bilayers. We found that heparin and other polyanions activated the Ca-gated Ca channel.

Further analysis of these effects of polyanions on the channels provides insights into the mechanism of action on the two different intracellular channels. It has been shown previously that heparin is a competitive inhibitor of the IP₃-gated channel (Ghosh *et al.*, 1988). We found that the action of the polyanions on the Ca-gated Ca channel is Ca dependent. A plausible model for this effect is that binding of a polyanion to the Ca-gated Ca channel increases the density of negative charges near a Ca-binding site which will lead to an increase in the local concentration of Ca at this regulatory site on the channel. Thus, the polyanions act by different mechanisms to induce opposite effects on the two different intracellular Ca release channels. As these two classes of Ca release channels coexist within cells, experiments that have used these compounds to separate the contribution of the two classes of channels in the regulation of intracellular Ca may be open for reinterpretation.

METHODS

Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared as described by Kim *et al.* (1983). Channel incorporation into planar lipid bilayers was accomplished as outlined previously (Smith *et al.*, 1986; Ehrlich and Watras, 1988), except that a KCl gradient was used for vesicle fusion. Final solutions used for channel analysis were as described previously (*cis* solution: 250 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]-tris(hydroxymethyl)-aminomethane [Tris], 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA], 0.5 mM CaCl₂ (80 nM free Ca), pH 7.3; *trans* solution: 250 mM HEPES, 53 mM Ca(OH)₂, pH 7.3).

Channel insertion and subsequent experiments were monitored under voltage-clamp conditions with a pair of Ag/AgCl electrodes contacting the solutions via CsCl junctions. The channel currents were amplified using a patch clamp amplifier (Patch Clamp model, Warner Instruments, Hamden, CT, or Axopatch 1-B, Axon Instruments, Foster City, CA) and recorded on chart (General Scanning, Watertown, MA) and tape recorders (Dagan Corp., Minneapolis, MN). Data were filtered with an 8 pole Bessel filter (Frequency Devices, Haverhill, MA) to 500 Hz, transferred to a personal com-

puter (digitized at 1.5 kHz) and analyzed using PClamp version 5.5 (Axon Instruments). Channel recordings are representative samples taken from ≥ 3 min of continuous records at each experimental condition. Except where noted, one of at least three similar experiments is shown. Data are expressed as means $\pm$ SE.

All reagents were of analytical grade or higher. Polysulfated compounds were purchased from Sigma (St. Louis, MO), Boehringer (Indianapolis, IN), and Calbiochem (San Diego, CA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Free Ca was determined as described previously (Fabiato, 1988).

RESULTS

In most cells, both IP₃- and Ca-gated Ca channels are present in the reticular membranes, although the relative density varies greatly among cell types. To ascertain if heparin and other polyanions affect the Ca-gated Ca channel, skeletal muscle "heavy" sarcoplasmic reticulum vesicles were employed. These heavy sarcoplasmic reticulum vesicles are enriched in Ca-gated/cafeine-gated Ca channels, identified as the ryanodine receptor (Smith *et al.*, 1988) and the density of IP₃-gated channels appears to be very low in this preparation (Watras and Benevolensky, 1987).

To determine whether polyanion-induced Ca release (Ritov *et al.*, 1985) was a reflection of channel activation, skeletal muscle sarcoplasmic reticulum vesicles were incorporated into planar lipid bilayers for analysis of Ca channel activity. As shown in Figure 1A, few channel openings were observed in the presence of 80 nM free Ca in the *cis* chamber (open probability $<0.2\%$; top trace). Addition of 1 $\mu\text{g/ml}$ heparin or 500 $\mu\text{g/ml}$ pentosan polysulfate (Figure 1A, second and third traces, respectively) to the *cis* chamber resulted in marked activation of a Ca channel. Similar results were obtained with other polyanions such as polyvinyl sulfate (10 $\mu\text{g/ml}$). Channel activation was observed in 14 of 15 experiments when heparin was tested and 11 of 12 experiments when pentosan polysulfate was tested.

The current amplitude and opening kinetics of this channel were similar to channels measured after activation by either Ca or caffeine under similar experimental conditions (Figure 1B; Smith *et al.*, 1986; Ehrlich and Watras, 1988). Addition of 5 μM ryanodine to the *cis* chamber modified the channel by reducing the single-channel conductance and slowing the kinetics (Figure 1A, bottom trace), consistent with the hypothesis that heparin is activating the ryanodine receptor/Ca channel.

Polyanion activation of this Ca channel was Ca dependent. The open probability of the skeletal sarcoplasmic reticulum Ca channel was very low in the presence of 5 nM Ca in the *cis* chamber (Figure 2, A and B, top traces). Addition of heparin (1 $\mu\text{g/ml}$) did not increase the open probability of this channel at this low

Figure 1. Activation of the Ca-gated Ca channel from skeletal muscle sarcoplasmic reticulum by polyanions. (A) At 80 nM free Ca (pCa 7.1; *cis* chamber), the Ca channel opened rarely ($p_o < 1\%$; top trace). Upon addition of 1 $\mu\text{g}/\text{ml}$ heparin or 500 $\mu\text{g}/\text{ml}$ pentosan polysulfate to the *cis* chamber, activation of the Ca channel was observed (second and third traces, respectively). Subsequent addition of ryanodine (5 μM) in the presence of 1 $\mu\text{g}/\text{ml}$ heparin modified the channel kinetics and current amplitude (bottom trace); similar results were obtained with channels activated by pentosan polysulfate. (B) Current-voltage relationship for the Ca-gated Ca channel from skeletal muscle. The slope conductance for the channel was 85 pS when activated by 0.5 μM Ca (●), 0.5 $\mu\text{g}/\text{ml}$ heparin (■), or 200 $\mu\text{g}/\text{ml}$ pentosan polysulfate (▼).

free Ca concentration (Figure 2A, second trace). Similarly, addition of 1 mg/ml pentosan polysulfate did not activate the channel at this Ca concentration (Figure 2B, second trace). When the free Ca concentration was increased above 80 nM in the presence of heparin or pentosan polysulfate, marked activation of the Ca channel was observed (Figure 2, A and B, third traces). Note that at a Ca concentration of 80 nM, the open probability of the channel is $<1\%$ in the absence of polyanion (Figure 1A; and Smith *et al.*, 1986). Subsequent addition of 2 μM ruthenium red (Figure 2, A and B, bottom traces) inhibited channel activity. When high concentrations of polyanion ($>1 \mu\text{g}/\text{ml}$) were used, 10–20 μM ruthenium red was needed to completely inhibit the channel in the bilayer, probably due to binding between the polyanion and ruthenium red (Ritov *et al.*, 1985).

The dependence upon Ca for the polyanion effect was observed at several concentrations of polyanion.

With a free Ca of $\leq 20 \text{ nM}$, neither heparin or pentosan polysulfate were able to activate the channel (Figure 3, A and B; ■). With 80 nM free Ca, polyanion addition induced a marked activation of the channel (Figure 3, A and B; ●). Half-maximal activation of the channel occurred at $0.23 \pm 0.01 \mu\text{g}/\text{ml}$ (mean $\pm$ SE) heparin, with a Hill coefficient of $4.1 \pm 0.9 \mu\text{g}/\text{ml}$ ($n = 3$; Figure 3A). Similar results were obtained with pentosan polysulfate ($k_{0.5} = 162 \pm 55 \mu\text{g}/\text{ml}$; Hill coefficient = 3.0 ± 0.6 , $n = 4$; Figure 3B).

Channel activity in the presence of polyanions could be increased or decreased by appropriately altering the free Ca concentration. The increased activity of the channel by addition of Ca in the presence of polyanion is shown in Figure 2, as discussed above. Inhibition of channel activity by lowering the free Ca in the presence of polyanion is shown in Figure 4. When the free Ca

Figure 2. Activation of the skeletal muscle Ca-gated Ca channel by heparin and pentosan polysulfate is Ca dependent. At 5 nM free Ca (pCa 8.3; *cis* chamber), the Ca channel opened rarely ($p_o < 0.2$; top traces). Addition of 1 $\mu\text{g}/\text{ml}$ heparin (A) or 1 mg/ml pentosan polysulfate (B) to the *cis* chamber did not increase the open probability of the channel (second traces). Increasing the free Ca concentration to 80 nM (pCa 7.1) in the presence of polyanion, however, resulted in a dramatic activation of the Ca channel (third traces). Channel openings shown in both A and B exhibit multiple subconductance states. Subsequent addition of ruthenium red (2 μM) blocked the channel (bottom traces).

Figure 3. Concentration dependence of the effect of polyanions on the skeletal muscle Ca-gated Ca channel observed at two Ca concentrations. Data obtained from recordings like those shown in Figures 1 and 2. Curves represent the best fit of the Hill equation to the data using a nonlinear curve fitting routine (Sigmaplot 4.0). (A) Increasing concentrations of heparin were added to the *cis* chamber in the presence of 80 nM free Ca (pCa 7.1; ●) or 20 nM free Ca (pCa 7.7; ■). (B) Increasing concentrations of pentosan polysulfate were added to the *cis* chamber in the presence of 80 nM free Ca (pCa 7.1; ●) or 4 nM free Ca (pCa 8.4; ■). One of at least three similar experiments for each polyanion is shown.

was 80 nM, the Ca release channel could be activated by heparin or pentosan polysulfate (Figure 4, A and B, middle traces). Subsequent addition of EGTA to lower the free Ca below 10 nM dramatically reduced the open probability of the channel ($p_o < 1\%$; Figure 4, A and B, bottom traces).

DISCUSSION

In the present study, heparin was shown to open the Ca-gated Ca channel from sarcoplasmic reticulum. This is opposite to the effect of heparin on another intracellular Ca release channel, the IP_3 -gated channel; heparin inhibits the IP_3 -gated channel (Ehrlich and Watras, 1988; Ghosh *et al.*, 1988; Kobayashi *et al.*, 1988). These diverse effects, however, were not restricted to heparin, as other polysulfated compounds such as pentosan polysulfate and polyvinyl sulfate also activated the Ca-gated Ca channel, while inhibiting the IP_3 -gated Ca channel (Kaftan *et al.*, 1992). These results provide further functional evidence that the IP_3 - and Ca-gated Ca channels are different. It also raises some concern about the use of heparin and related compounds in studies aimed at elucidating the role of the IP_3 -gated Ca channel in various tissues. Therefore, one must be aware of the

potential problems associated with the use of heparin, as it can act as both an inhibitor of the IP_3 -gated Ca channel and an activator of the Ca-gated Ca channel.

Heparin has been shown to be a competitive inhibitor of IP_3 binding to purified IP_3 receptor (Supattapone *et al.*, 1988) and of IP_3 -induced Ca release from vesicles (Ghosh *et al.*, 1988). The observed Hill coefficient of 2.5–3.2 for inhibition of Ca release by polyanions raises the possibility that heparin must bind to at least three of the IP_3 receptors to inhibit the channel (Watras *et al.*, 1991b).

The Ca-gated Ca channel is also affected by heparin, polyvinyl sulfate, and pentosan polysulfate, but in a

Figure 4. The effect of Ca on the polyanion-induced activation of the skeletal muscle Ca-gated Ca channel is reversible. At 80 nM free Ca (pCa 7.1; *cis* chamber), the Ca channel opened rarely ($p_o < 1\%$; A and B, top traces). Upon addition of 5 μ g/ml heparin or 500 μ g/ml pentosan polysulfate to the *cis* chamber (A and B, respectively), activation of the Ca channel was observed (second traces). Subsequent addition of EGTA in the presence of heparin or pentosan polysulfate (A and B, respectively) inhibited channel openings (bottom traces).

very different way. In this case the presence of polyanions activates the channel. The concentration dependence of the polyanion effects on the Ca-gated Ca channel generates a Hill coefficient of 2–4. As the channel complex is a tetramer (Lai *et al.*, 1988; Wagenknecht *et al.*, 1989), this Hill coefficient could reflect association of a polyanion with each of the four subunits comprising the channel complex. Although the specificity of the interactions between the polyanions and the ryanodine receptor is unknown, the observed cooperativity suggests that several polyanion molecules need to interact with the Ca-gated Ca channel complex to affect channel activity.

A possible mechanism by which this polyanion-dependent activation of the Ca-gated Ca channel occurs, which is consistent with the available data, involves alteration in the Ca concentration near the regulatory site of the Ca channel. This hypothesis is based in part on the observation that Ca activates this channel at Ca concentrations > 100 nM (Smith *et al.*, 1986). In this model, association of the polyanions with the channel would produce an increase in localized negative charge, which could attract cations such as Ca. A local increase in Ca concentration in or near the Ca-gated Ca channel would activate this channel (Smith *et al.*, 1986; Bezprozvanny *et al.*, 1991). If this were the case, one might expect that addition of polyanions at very low levels of free Ca would not activate the Ca channel, whereas higher levels of Ca may potentiate the polyanion-dependent activation of the Ca channel. Consistent with this prediction, polyanion activation of the Ca-gated Ca channel incorporated into planar lipid bilayers was shown to be dependent on free Ca concentration (Figures 2–4). Specifically, in the presence of 80 nM free Ca, 1 μ g/ml heparin markedly activated the channel, whereas even higher concentrations of heparin did not activate the channel in the presence of 20 nM free Ca. The Ca dependence of the response of the Ca-gated Ca channel to polyanion addition may explain the difference in response to heparin addition seen in two previous reports (Ritov *et al.*, 1985; Palade *et al.*, 1989).

Most studies of the IP₃ receptor use heparin as a specific inhibitor. Pentosan polysulfate can replace heparin in assays of the IP₃ receptor function (Watras *et al.*, 1991b) and in activation of the Ca-gated Ca channel (this report). There are advantages of using pentosan polysulfate over heparin. Pentosan polysulfate is a polymer synthesized of known subunits and is thus a more precisely defined compound than heparin which is a family of compounds. We have found empirically that reversibility of channel activation by pentosan polysulfate is more reliably complete than that achieved with heparin.

In summary, the present study shows that heparin activates the Ca-gated Ca channel in the sarcoplasmic reticulum whereas, previous studies have shown that

it inhibits the IP₃-gated Ca channel. These results provide further functional evidence that these channels are different. Heparin and a few other polyanions alter IP₃-gated Ca channel function by competitive inhibition of IP₃ binding, with binding to at least three subunits of the IP₃ receptor complex necessary to block the channel (Watras *et al.*, 1991b). A mechanism by which polyanion-induced activation of the Ca-gated Ca channel may occur is by association with the receptor and subsequent increase in the local concentration of Ca near the regulatory site of the channel due to electrostatic interactions.

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