Block of ATP-sensitive K⁺ channels in isolated mouse pancreatic β-cells by 2,3-butanedione monoxide

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Introduction

2,3-Butanedione monoxide (diacetyl monoxide, BDM) is a well established chemical phosphatase. In cardiac myocytes and dorsal root ganglion neurones, extracellularly applied BDM has been shown to inhibit L-type Ca²⁺-channels (IC₅₀ = 5.8 mM, Chapman, 1993; IC₅₀ ≈ 20 mM, Huang & McArdle, 1992). Inhibition was reversed by agents that are thought to promote phosphorylation of the L-type calcium channel, such as β-bromo-cyclic AMP and isoprenaline. These data are consistent with the idea that BDM can behave as a chemical phosphatase and can decrease L-type Ca²⁺-channel activity by dephosphorylation of the channel protein (Huang & McArdle, 1992; Chapman, 1993). In ventricular myocytes, BDM reduces both the slow inward calcium current and the transient outward potassium current, effects which have again been attributed to a phosphatase action of BDM (Coulombe et al., 1990). BDM has also been shown to inhibit both ATP-sensitive K⁺-channels (K_ATP-channels) in cardiac myocytes, K ≈ 21 mM, and voltage-dependent K⁺-currents expressed in oocytes (Hardin et al., 1993; Lopatin & Nichols, 1993). In these cases, however, inhibition was reversible in the absence of Mg²⁺ ions and ATP, conditions which do not support protein phosphorylation. This suggests that BDM may also block channel activity by a mechanism that does not involve protein dephosphorylation. We have used standard patch-clamp techniques to investigate whether BDM acts as a direct blocker of K_ATP-channels in mouse pancreatic β-cells.

Methods

Cells

Patch-clamp studies were made on primary cultured β-cells isolated from mouse islets of Langerhans, as previously described (Rorsman & Trube, 1985; Bokvist et al., 1990).

Solutions

In whole-cell and outside-out patch experiments the extracellular face of the membrane was bathed in a solution containing (in mM): NaCl 138, KCl 5.6, CaCl₂ 2.6, MgCl₂ 1.1, Na-HEPES 10 (pH 7.4). For the inside-out experiments the extracellular, pipette, solution contained (in mM): KCl 140, CaCl₂ 2.6, MgCl₂ 1.2, K-HEPES 10 (pH 7.4). In all experiments the intracellular face of the membrane was bathed in a solution which contained (in mM): KCl 140, CaCl₂ 4.6, EDTA 10, K-HEPES 10 (pH 7.2; free [Mg²⁺] < 6 nM; free [Ca²⁺] < 30 nM). This solution does not support protein phosphorylation since it does not contain Mg²⁺ or ATP. It also reduces rundown of K_ATP-channels in excised patches and standard whole-cell recordings from pancreatic β-cells (Kozlowski & Ashford, 1990; Williams, 1992). All chemicals were from Sigma. 2,3-Butanedione monoxide (BDM) was dissolved directly into the appropriate solution. All experiments were carried out at room temperature (23–26°C).

Electrophysiological recording

Membrane-currents were recorded with a List EPC-7 amplifier (List Electronik, Darmstadt, Germany), filtered at 2–5 kHz (–3 dB) with an 8 pole Bessel filter (Frequency devices, Burlingame, MA, U.S.A.) and subsequently digitized at 5–10 kHz using AXOLAB hardware (Axon Instruments, Foster City, CA, U.S.A.) and analysed using in-house software.

Whole-cell currents flowing through K_ATP-channels were measured according to the protocol of Trube et al. (1986), that is as the current elicited by alternate ± 10 mV voltage steps (200 ms duration at 0.5 Hz) from a holding potential of −70 mV.

In inside-out patch experiments single K_ATP-channel currents were recorded at a membrane potential of −70 mV. For outside-out patch studies the membrane potential was held at 0 mV; although this potential lies above the threshold.
for activation of both the delayed-rectifier and calcium-activated K-channels (KCa-channels), these channels will be substantially inactivated by the maintained depolarization. Furthermore, the free Ca$^{2+}$ concentration in our intracellular solution is below that required for activation of KCa-channels (Smith et al., 1990).

Data analysis

To determine the degree of KATP-current inhibition, the whole-cell KATP-current (I) measured in the test solution was normalized to the average ($I_e$) of its value in the control solutions preceding, and following, exposure to the test solution. The data were fitted with the Hill equation:

$$\frac{I}{I_e} = \frac{1}{1 + ([\text{BDM}]/K)^n}$$

where $K$ is the concentration at which half maximal inhibition occurs and $n$ is the Hill coefficient.

When measuring channel activity (NP) we did not attempt to discriminate between changes in the number of available channels, $N$, and changes in the channel open probability, $P$. NP was defined by $(\Sigma z_j t_j)/T$, where $T$ is the total time of the recording, $t_j$ is the total time for which the $j$th channel is open, and $n$ is the maximum number of channels observed in the patch. The relationship between BDM concentration and channel activity was determined by normalizing the channel activity in the test solution (NP) to the average of its value in the control solution before and after exposure to BDM (NPc). The data were then fitted by the Hill equation (Equation 1).

Single-channel currents were analysed using the half-amplitude threshold technique following the methods detailed by Colquhoun & Sigworth (1983). Lifetime distributions were log-binned using the method of McManus et al. (1987) where:

$$\text{bin} = 1 + \text{integer}(25 \times \log_{10}(\text{event duration in samples}))$$

When the square root of the number of events in a bin is plotted against the open or closed lifetime, the components of the distribution appear as clear peaks with their respective time constants falling in the vicinity of the distribution peaks (Sigworth & Sine, 1987). Conditional probability density functions (PDF) were fitted to the open, closed and burst lifetime distributions by the method of maximum likelihood. We used the following PDF:

$$f(t) = \sum_{i=1}^{k} a_i \frac{1}{P(t_{min} < t < t_{max})} \exp(-t/t_i)$$

where:

$$P(t_{min} < t < t_{max}) = \sum_{i=1}^{k} a_i \left(\exp(-t_{min}/t_i) - \exp(-t_{max}/t_i)\right)$$

and $a_i$ is the relative area, and $t_i$ the time constant, of the $i$th component. $t_{min}$ and $t_{max}$ define the shortest and longest observations used in fitting the PDF. No correction was made for missed events.

In patches where multiple channel openings were present, we have limited our kinetic analysis to that of the open and closed times within bursts of channel openings during periods when only a single-channel was active. In patches in which channel activity was low or was dramatically reduced by BDM, we were able to perform a kinetic analysis of the burst of channel openings. A burst was defined as one or more openings which were separated by closures which were less than the burst criteria time. $t_{on}$ - $t_{off}$ was determined from:

$$a_i [\exp(-t_{off}/\tau_{c}) - \exp(-t_{on}/\tau_{c})] = a_{c,f} \exp(-t_{on}/\tau_{c,f})$$

where $a_{c,f}$ and $\tau_{c,f}$ are the relative area and time constant of the fastest component of the closed times, and $a_{c,i}$ and $\tau_{c,i}$ are the relative area and time constant of the intermediate component of the closed times (Jackson et al., 1983). Newton’s method was used to find the implicit variable, $t_{on}$. Long closed times composing the $\tau_{c,i}$ component were excluded from this calculation as they occurred too infrequently to substantially affect $t_{on}$.

Values are quoted as mean ± s.e.mean, $n$ = number of cells.

Results

Effects of BDM on whole-cell $K_{ATP}$ currents

Following establishment of the whole-cell configuration, the $K_{ATP}$-current rapidly increased, due to washout of ATP from the cell into the pipette (Trube et al., 1986), reaching a maximum within 2–3 min. The subsequent decline (run-down) of $K_{ATP}$-currents is markedly reduced when divalent cations in the intracellular solution are buffered to nanomolar concentrations (Kozlowski & Ashford, 1990; Williams, 1992) and with our intracellular solution we found that the $K_{ATP}$-current remained stable for periods in excess of 30 min. The effect of BDM was tested only after the whole-cell $K_{ATP}$

![Figure 1](image_url)
currents had reached a stable level. As shown in Figure 1a, the addition of BDM to the extracellular solution caused a rapid and reversible reduction of both the whole-cell KₐTP-
current and the holding current. The relationship between
BDM concentration and the whole-cell KₐTP-current (Figure
1b) was well fitted by the Hill equation (Equation 1) with a
Kᵣ of 14 mM and a Hill coefficient of 2.2. Individual fits to
four complete dose-response curves gave mean values for Kᵣ
of 15 ± 1 mM and of 2.5 ± 0.2 for the Hill coefficient. Whole-
cell KₐTP-currents were unaffected by the addition of 60 mM
sucrose to the bath, indicating that the block by high BDM
concentrations does not result from the increase in osmotic
strength.

Effect of external BDM on single KₐTP-channel currents in outside-out patches

Channel activity In order to study the mechanism of block
in more detail, we examined the action of BDM on single
KₐTP-channel currents in outside-out patches held at 0 mV.
Increasing the concentration of external BDM reduced the
activity of the KₐTP-channel but had little effect on the
single-channel current amplitude (Figure 2a). Figure 2b
shows the relationship between BDM concentration and
channel activity. The fractional channel activity (NP/NPC)
was fitted by the Hill equation (Equation 1) with a Kᵣ
of 11 ± 3 mM and a Hill coefficient of 2.0 ± 0.3 (n = 7). Inhibi-
tion was almost complete at 60 mM BDM with only
2.0 ± 1% (n = 5) of channel activity remaining. Sucrose
(30 mM) had no effect on channel activity, single-channel
current amplitude or single-channel kinetics, supporting the
idea that the action of external BDM is not osmotic in
origin.

Kinetics of block Openings of the KₐTP-channel are grouped
into bursts, which consist of a series of consecutive channel
openings separated by fast closures. BDM reduced the burst
duration in a dose-dependent manner (Figures 2a and 2b).
It also decreased both the number of the openings within a
burst and the mean open lifetimes (Figures 3 and 4a).

The distribution of open lifetimes of the KₐTP-channel at
0 mV (Figure 3) was best described by a PDF consisting of
the sum of two exponentials (Equation 3, k = 2). This is
indicated by the solid line in Figure 3a. In control solution,
the time constant of the fast component (τₕ₉) illustrated
was 0.21 ms (mean 0.19 ± 0.01 ms, n = 5) and that of the slower
component (τₛ₉) was 3.1 ms (mean 3.7 ± 0.4 ms, n = 5).
BDM had little effect on τₛ₉ but greatly reduced τₕ₉ in
this particular patch, 30 mM BDM reduced τₕ₉ from 3.1 to 1.9 ms
(Figure 3c). In five patches 30 mM BDM reduced τₕ₉ by 70% to
1.1 ± 0.3 ms, but we could not detect a difference in τₛ₉,
which remained at 0.24 ± 0.1 ms. Although the relative areas
(see Methods) of the two open lifetime components differed
between patches they were unchanged by BDM (Figure 3a,c).

The decrease in τₕ₉ by BDM is reminiscent of the block of
KₐTP-channels produced by external Ba⁺⁺ (Quayle et al.,
1988) and suggests the following reaction scheme for the
action of external BDM:

\[ C \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \quad \text{open} \quad \underset{k_{-k_{-1}}}{\overset{k_{-1}}{\rightleftharpoons}} \quad B \quad \text{(Scheme 1)} \]

where O represents the open state associated with τₛ₉, the
long open time component, C is the sum of the adjacent
closed states and B is the blocked state produced by BDM.
This scheme predicts that the reciprocal of τₛ₉ will be given by:

\[ \frac{1}{\tau_{s_{9}}} = k_{-1} + k_{-k_{-1}} \quad \text{[BDM]} \]

(6)

where \( k_{-1} = 1/\tau_{s_{9}} \) in the absence of BDM. Figure 4a shows a
representative plot of 1/τₛ₉ against BDM concentration. The
solid line is the best fit of Equation 6 to the data with
\( k_{-k_{-1}} = 8.4 \text{mM}^{-1} \text{s}^{-1} \) and \( n = 1.6 \), taking τₛ₉ to be 4.6 ms in
the absence of BDM (the value measured in the patch illus-
trated). Mean values for \( k_{-k_{-1}} \) of 9.0 ± 0.5 mM⁻¹s⁻¹ and of
1.5 ± 0.1 for n were obtained in three patches.

The distribution of closed times was described by a PDF
consisting of the sum of three exponentials (Equation 3,
\( k = 3 \)), representing the short, intermediate and long closures
and having time constants \( \tau_{c_{1}}, \tau_{c_{2}} \) and \( \tau_{c_{3}} \) respectively.
The fast closures separating openings within bursts are defined
by \( \tau_{c_{1}} \) while \( \tau_{c_{2}} \) and \( \tau_{c_{3}} \) represent the closures that separate
bursts of openings. The presence of two components of
closures between bursts suggest that the bursts are grouped
together in clusters. In control solution (Figure 3b), time
constants of 0.2 ms for \( \tau_{c_{1}} \), 2.8 ms for \( \tau_{c_{2}} \) and 13 ms for \( \tau_{c_{3}} \),

Figure 2 Effect of BDM on single KₐTP-channel currents in an
outside-out patch held at 0 mV. (a) Single KₐTP-channel currents
recorded at the indicated concentrations of external BDM. The
dashed line indicates the channel closed level. Data filtered at 1 kHz
for display. (b) Dose-response relationship of external BDM on the
channel activity (NP) normalized to that in the absence of the drug
(NPc). Each point is the mean from the number of experiments
indicated in parentheses. Vertical bars indicate s.e.mean except when
this is no larger than the symbol. The solid line is a fit to the Hill
equation with a \( Kᵣ \) of 10 and a Hill coefficient of 1.4.
were obtained at 0 mV. BDM (30 mM) had no effect on \( \tau_{o-f} \) (mean value 0.20 ± 0.05 ms in the absence and 0.19 ± 0.04 ms in the presence of 30 mM BDM, \( n = 5 \)). BDM, however, greatly prolonged the two slower time constants, \( \tau_{s} \) and \( \tau_{o-f} \); in this particular patch these increased to 5.5 ms and 40 ms respectively (Figure 3d).

Burst of \( K_{ATP} \)-channel openings were analysed using a burst criteria time, \( t_{on} \), calculated for each experimental condition from Equation 5. The distribution of the burst lengths were fitted with a PDF consisting of the sum of two exponential components, comprising the short and long bursts (Equation 3, \( k = 2 \)), with mean lengths, \( t_{o-s} \) and \( t_{s-d} \) respectively. In both the presence and absence of BDM, the mean lifetime of the short burst, \( t_{s-d} \), was almost identical to that of the mean fast open time, \( \tau_{o-f} \) (\( t_{o-s} = 0.22 ± 0.04 \) ms, \( \tau_{o-f} = 0.19 ± 0.01 \) ms, \( n = 5 \) in control solution). Furthermore, the short bursts possessed a mean of one opening per burst (1.03 ± 0.02, \( n = 5 \)) which suggest that the short bursts are simply single openings of the \( K_{ATP} \)-channel to the short open state (\( \tau_{o-s} \)). A similar phenomena has been described in skeletal muscle (Spruce et al., 1985). The lifetime of the short bursts did not appear to be affected by 30 mM BDM (mean 0.23 ± 0.07 ms, \( n = 5 \), consistent with the lack of effect of the drug on \( \tau_{o-f} \).

The longer component of burst lengths consisted of several openings per burst. Both the mean burst length, \( t_{o-s} \) and the number of openings per burst decreased with increasing BDM concentration. In five patches the mean burst length was 19 ± 5 ms in control and 4.0 ± 0.9 ms in the presence of 30 mM BDM. The mean number of openings per long burst was 5.0 ± 0.7 in the absence of BDM and was reduced to 3.9 ± 0.4 in the presence of the drug (\( n = 7, \ P = 0.016 \)).
Figure 4b illustrates a plot of $1/t_{0.5}$ versus BDM concentration. The solid line is a fit to the data of:

$$\frac{1}{t_{0.5}} = k_{-2} + k_{h2} [\text{BDM}]^n$$  \hspace{1cm} (7)$$

with $k_{h2} = 0.44 \text{mM}^{-1} \text{s}^{-1}$ and $n = 1.8$ (mean $k_{h2} = 0.66 \pm 0.12 \text{mM}^{-1} \text{s}^{-1}$ and $n = 1.9 \pm 0.1$, $n = 3$). Equation 7 is derived from the following reaction scheme:

$$\text{C} \quad \xleftarrow{k_{-2}} \quad \text{O-O} \quad \xrightarrow{k_{h2}} \quad \text{B} \quad \quad \text{(Scheme 2)}$$

where O-O represents openings that collectively form a burst, C is the sum of the interburst closed states and B is the blocked state produced by BDM. In the absence of BDM, $k_{-2} = 1/t_{0.5}$, where $t_{0.5}$ was 30 ms in the example illustrated in Figure 4b.

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Figure 5 Effect of internal BDM on single KATP-channel currents recorded from an inside-out patch held at $-70 \text{ mV}$. The dashed line indicates the channel closed level. (a) Control; (b) 30 mM BDM; (c) 60 mM BDM; (d) channel activity, NP, normalized to that in the absence of the drug, NPC, plotted against the internal BDM concentration. Each point is the mean from the number of experiments indicated in parentheses. Vertical bars indicate s.e.mean except when this is no larger than the symbol. The solid line is a fit to equation 1 of the text with $K_c = 31 \text{ mM}$ and $n = 1.7$.

Figure 6 Effect of internal BDM on KATP-channel kinetics. Open times (a and c) and closed times (b and d) are log-binned according to equation 2. The solid lines are fits to the appropriate PDF using equation 3, as described in the text. (a) and (b) are the open time and closed time distributions in the absence of BDM; (c) and (d) are the open time and closed time distributions from the same patch in the presence of 30 mM internal BDM. See text for fitted parameters.
Voltage-dependence of block The block by BDM was voltage-independent, since neither the reduction in channel activity (NP/NPe = 0.17 at -30 mV) nor the changes in channel kinetics caused by 30 mM BDM varied with the holding potential. Furthermore, 30 mM BDM had little effect on the single-channel current amplitude or the variance of the open-channel current at potentials between -30 mV and +30 mV. The similarity between the K_i for channel inhibition determined in whole-cell (15 mM) and outside-out patch (11 mM) recordings further supports the idea that the block by external BDM is voltage-independent since the holding potentials were -70 mV and 0 mV respectively in these configurations.

Effect of intracellular BDM on single K_ATP-channels in inside-out patches

Channel activity When applied to the cytosolic face of an inside-out patch, BDM rapidly and reversibly reduced the activity of the K_ATP-channel. Figure 5a-c shows single-channel currents recorded at different BDM concentrations and Figure 5d the relationship between channel activity and BDM concentration. The data were fitted to Equation 1 with a mean K_i of 31 ± 2 mM and a Hill coefficient of 2.0 ± 0.4 (n = 8). The effect of internal BDM on K_ATP-channels was thus less potent than that of externally applied BDM where the K_i was 11 mM.

Internal BDM did not affect the single-channel current amplitude (Figure 5a, b and c).

Kinetics The kinetics of the K_ATP-channel in the inside-out patch differed from those in the outside-out patch. This difference may be explained by the different holding potentials used (Spruce et al., 1985; Davies et al., 1989), by the different K^+ gradients used or by both, that is by differences in the electromotive force (Zilberter et al., 1988). At -70 mV, the distribution of open lifetimes of the K_ATP-channel in the absence of BDM was described by a PDF that consisted of only one exponential component (Equation 2, k = 1) with a single time constant, τ_p. A representative open time distribution in control solution is shown in Figure 6a; 30 mM BDM had only a minor effect on the open channel lifetime, reducing τ_p by 14% from 1.8 ± 0.1 ms (n = 8, Figure 6a) to 1.6 ± 0.1 ms (n = 4, Figure 6b).

Although channel openings were grouped into bursts, the bursts did not appear to exhibit clustering behaviour, the distribution of closed times being best described by a PDF consisting of the sum of two exponentials (Equation 3, k = 2). Representative examples are shown in Figure 6b and d. The short closures (gaps) within bursts and the long closures between bursts are represented by time constants, τ_c and τ_a respectively.

BDM had no effect on τ_c, which had a mean value of 0.41 ± 0.01 ms (n = 8) in control solution and of 0.40 ± 0.02 ms in the presence of 50 mM BDM (n = 5). Due to the high level of channel activity in the majority of inside-out patches it was not possible to obtain an accurate measure of the long closed time constant, τ_L, in control solution, but it was apparent from the records that BDM caused a graded reduction in burst length (Figure 5a-c).

Discussion

We show here that the block of K_ATP-channel activity by BDM is fully reversible under non-phosphorylating conditions in both whole-cell and excised patch recordings. This indicates that BDM blocks channel activity by a mechanism unrelated to its action as a phosphatase and instead functions as a direct blocker of the K_ATP-channel. Similar findings have been reported for ventricular myocytes, where BDM causes a rapid and reversible block of the K_ATP-channel in the inside-out patch configuration with a K_i of 21.3 mM and a Hill coefficient of 1.8 (Hardin & Nichols, 1992). These values are very close to those that we observe (K_i = 31 mM and n = 2) in the same patch configuration. The higher potency of BDM in blocking ventricular calcium currents (IC_50 = 5.8 mM, Chapman, 1993) may reflect a phosphatase action, as a shift in the potency of block by BDM occurs with conditions that would be expected to affect both the degree and stability of channel calcium phosphorylation (Chapman, 1993). We have not explored any possible actions of BDM under phosphorylating conditions.

The ability of BDM to act as a chemical phosphatase has often been exploited to investigate the role of protein phosphorylation in the regulation of ionic channel activity. Our results imply, however, that it is essential to exclude the possibility that the drug also acts as a direct channel blocker in such studies.

The ability of BDM to reduce the open-channel lifetime, the burst duration and the channel open probability without affecting either the single-channel current amplitude or the fast closed time is reminiscent of the actions of barium (Quayle et al., 1988) and tolbutamide (Gillis et al., 1989) on this channel and suggests that like these agents BDM behaves as a 'slow' blocker according to the nomenclature of Hille (1992).

Although BDM is more lipophilic, whereas octanol partition coefficient of 16 (Leo et al., 1971), our data suggest that the drug may have both an external and internal site of action. The K_i for channel inhibition by external BDM was 11 mM in outside-out patches and 15 mM in whole-cell recordings suggesting that BDM acts at an extracellular site in the latter case. Internal BDM was less effective at inhibiting channel activity, the K_i being 31 mM. This lower sensitivity to internal BDM cannot be explained by the difference in holding potential between inside-out (-70 mV) and outside-out patches (0 mV) since the whole-cell currents were also recorded at a holding potential of -70 mV. Furthermore, the effects of both internal and external BDM are voltage-independent. The effect of BDM on the channel kinetics was also dependent on whether the drug was applied to the external or internal face of the channel. Thus extracellular applied BDM markedly reduced the longer of the two channel open lifetimes, whereas BDM had only a minor effect on τ_c when applied intracellularly.

An alternative explanation of the lower efficacy of BDM when applied intracellularly, is that there is a single site of action for BDM the affinity of which varies with the extracellular K^+ concentration. In this model external K^+ ions would allosterically reduce the binding of BDM, either by acting at an extracellular binding site or within the pore. This would explain why the K_i for BDM inhibition is lower in inside-out patches (where the concentration of K^+ is 11 mM) than in outside-out patches, or from whole-cell recordings, where the concentration of K^+ is only 5 mM.

The Hill coefficient for both external and internal block was greater than one and suggests the cooperative action of more than one BDM molecule is required to inhibit the channel.

Our analysis of the kinetics of the K_ATP-channel in outside-out patches from β-cells is the first to be documented at a high temporal resolution. We observed that the channel possesses both a short and a long lived open state with mean lifetimes of 0.19 ms and 3.7 ms respectively. These openings occur in bursts, with a mean intraburst closed time of 0.2 ms, and there is an excess of bursts in which only a single opening is present. Similar kinetics have been described for inward K_ATP-channel currents in inside-out patches from frog skeletal muscle by Spruce et al. (1985).

We attempted to quantify the effects of BDM on channel kinetics using an empirical model in which the blocked state represents a new closed state entered directly from the open state. A model in which BDM facilitates entry into an existing closed state can also explain our results and would yield the same rate constants. Theoretically these two models may be distinguished because the former predicts the existence of
an additionally closed state, that produced by BDM block. No additional closed state was observed in the presence of BDM in our experiments. In practice however, a new closed state may not be resolved if it is of a duration similar to that of the closed states existing in the absence of BDM.

Therefore it is not possible to distinguish between the two models.

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References


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