A study of NPY-mediated contractions of the porcine isolated ear artery


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1 Enhanced contractions of the porcine isolated ear artery by the $z_2$-adrenoceptor agonist UK14304 are uncovered by pharmacological manipulation. As both neuropeptide Y (NPY) receptors and $z_2$-adrenoceptors are negatively-coupled to adenylyl cyclase in this tissue, we determined whether NPY is also able to produce an enhanced contraction in the same tissue, under the same conditions.

2 NPY (0.1 $\mu$M) produced a small contraction of porcine isolated ear arteries which was $5.1 \pm 0.8\%$ of the response to 60 mM KCl ($n=14$). An enhanced NPY response was uncovered if the tissue was pre-contraction with 0.1 $\mu$M U46619, and relaxed back to baseline with 1–2 $\mu$M forskolin before the addition of NPY (49.8 ± 5.3%, $n=14$).

3 Forskolin (1 $\mu$M) stimulated cyclic AMP accumulation in porcine ear artery segments in the presence of 0.1 $\mu$M U46619 and 1 mM isobutylmethylxanthine (IBMX), NPY (0.1 $\mu$M) inhibited this response by 40%, but had no effect on basal levels of cyclic AMP.

4 An enhanced response to 0.1 $\mu$M NPY was also obtained after pre-contraction with 0.1 $\mu$M U46619 and relaxation with either SNP (28.9 ± 5.7%, $n=14$), or dibutyl cyclic AMP (21.2 ± 4.6%, $n=14$). This indicates that at least part of the enhanced response to NPY is independent of the agonist’s ability to inhibit adenylyl cyclase.

5 In conclusion, an enhanced contraction to NPY in the porcine isolated ear artery can be obtained by prior pharmacological manipulation. The enhanced responses are mediated through adenylyl-cyclase-dependent and independent pathways similar to those reported for $z_2$-adrenoceptors in this preparation.

**Keywords:** Neuropeptide Y; porcine ear artery; vasoconstriction; cyclic AMP; sodium nitroprusside; UK14304; $z_2$-adrenoceptors

**Abbreviations:** AII, Angiotensin II; ANOVA, analysis of the variance; IBMX, isobutylmethylxanthine; NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; SNP, sodium nitroprusside; UK14304, 5-bromo-6-[2-imidazolin-2-ylamine]-quinoxaline bitartrate

**Introduction**

Neuropeptide Y (NPY) is a member of a family of structurally related peptides, including peptide YY (PYY) and the pancreatic polypeptide (PP), which produce a wide range of effects throughout the body. Based on molecular biological and pharmacological criteria, five receptors at which these peptides act have been identified so far, designated Y₁–Y₅ (see Balasubramaniam, 1997 for review). The majority of the cardiovascular effects of NPY appear to be mediated via the NPY Y₁ receptor (see McDermott et al., 1993 for review). However, in some vascular beds there is evidence that responses may also involve the NPY Y₂ receptor subtype (Tessel et al., 1993; Pheng et al., 1997). In the vasculature, NPY is generally considered to have a vasoconstrictor effect through either a direct or an indirect mechanism. It produces small vasoconstrictor responses in isolated cerebral arteries from the cat (Fredholm et al., 1985), rat (Xia et al., 1992), and rabbit (Abel & Han, 1989), and in the isolated coronary artery from rabbit (Han & Abel, 1987). On the other hand, NPY potentiates responses to a variety of vasoconstrictors in tissues in which it has little or no direct vasoconstrictor effect (Abel & Han, 1989; Han & Abel, 1987; Wahlestedt et al., 1985; Xia et al., 1992). For example, NPY enhanced both the contraction and the associated inositol phosphate response to angiotensin II in the rabbit femoral artery (Cressier et al., 1995). Thus, NPY appears to modulate vascular responses to other agonists, possibly through an interaction at the second messenger level, in target vessels.

NPY activates a number of intracellular responses in isolated blood vessels and vascular smooth muscle cells maintained in culture, including an inhibition of adenosine 3’:5’-cyclic monophosphate (cyclic AMP) production (Fredholm et al., 1985; Prieto et al., 1997), an increase in [Ca²⁺]$^+$ (Erdbrugger et al., 1993; Mihara et al., 1989), and membrane depolarization (Prieto et al., 1997). However, the precise relationship between these responses and NPY-induced vasoconstriction is unclear. For example, preliminary studies in our laboratory have shown that NPY inhibits forskolin-stimulated cyclic AMP accumulation in the isolated thoracic aorta, ear artery, and ear vein from the pig (Wright et al., 1995), yet significant vasoconstrictor responses to NPY were only observed in the ear vein. This indicates that there is no direct relationship between the NPY-mediated inhibition of cyclic AMP accumulation and vasoconstriction in porcine blood vessels, a situation which is similar to that for vascular $z_2$-adrenoceptors (Wright et al., 1995).

In the porcine isolated ear artery 5-bromo-6-[2-imidazolin-2-ylamine]-quinoxaline bitartrate (UK14304), a selective $z_2$-adrenoceptor agonist, inhibits adenyl cyclase, but the vasoconstrictor responses are small (Roberts et al., 1998). However, pre-contraction of the tissue with the thromboxane-mimetic U46619 followed by relaxation with a cyclic...
nucleotide-generating agent, or a cell permeable analogue, causes an enhancement of vasoconstrictor responses to UK14304. The enhanced responses appear to be mediated through both adenyl cyclase-dependent and -independent pathways. In view of the qualitative similarities between vascular \( \alpha \)-adrenoceptors and NPY receptors, it was of interest to determine whether similar pharmacological manipulations could also enhance NPY-induced contractions in the porcine isolated ear artery.

**Methods**

**Functional studies**

Isometric tension recordings Porcine ears were obtained from a local abattoir and transported to the laboratory on ice. Ear arteries were dissected out and placed in Krebs-Henseleit buffer containing 2% ficoll, which had been pre-gassed with 95% O\(_2\)/5% CO\(_2\), and stored overnight at 4°C (see Wright et al., 1995a). The following day ear arteries were carefully cleaned of fat and connective tissue, dissected into 5 mm ring segments, and suspended in a 5 ml isolated organ bath containing Krebs-Henseleit buffer maintained at 37°C and constantly gassed with 95% O\(_2\)/5% CO\(_2\). The lower support was fixed and the upper support was connected to a force transducer (World Precision Instruments, Sarasota, Florida, U.S.A.) linked to a Maclab data acquisition system (AD Instruments Ltd., Hastings, U.K.) via an amplifier. After a 20 min equilibration period, tension was applied to the tissue which was allowed to relax until a state of tension that varied between 1–1.5 g wt. Before each experiment the tissues were contracted at least three times with 60 mM KCl, until the final two responses differed by less than 10%. Between each response, tissues were washed three times with Krebs-Henseleit buffer and allowed to recover for 20 min.

Responses to NPY in the presence of angiotensin II and U46619 In order to compare the responses obtained with NPY with those obtained previously with the \( \alpha \)-adrenoceptor agonist UK14304, pharmacological manipulations using the same concentrations of pre-contracting and relaxing agents (see below) were performed (see Roberts et al., 1998).

Ear arteries were exposed to 30 nM angiotensin II (AII) which gave a transient contraction. After the tension had returned to baseline, 0.1 \( \mu \)M NPY was added. Responses to NPY after the addition of AII were measured from the pre-NPY baseline. Increases in the tone induced by NPY were measured from the pre-NPY baseline.

Effect of relaxing agents on subsequent NPY responses Ear arteries were contracted with 0.1 \( \mu \)M U46619 and relaxed to <10% of the 60 mM KCl response with forskolin (1–2 \( \mu \)M), sodium nitroprusside (SNP; 100–200 \( \mu \)M), or dibutyryl cyclic AMP (3–5 \( \mu \)M), before 0.1 \( \mu \)M NPY was added. Responses to NPY obtained under these conditions were compared to control responses in which 0.1 \( \mu \)M NPY was added to tissues on its own. Increases in the tone induced by NPY were measured from the pre-NPY baseline.

Effect of pre-contraction with UK14304 on subsequent responses to NPY Tissues were pre-contrasted with 0.1 \( \mu \)M U46619 and relaxed back to baseline with forskolin as above, before the addition of 0.3 \( \mu \)M UK14304. UK14304 responses were allowed to reach a plateau before the addition of 0.1 \( \mu \)M NPY. The responses to NPY under these conditions were compared to the responses to 0.1 \( \mu \)M NPY without pre-exposure to any other agent.

Cyclic AMP measurements Ear arteries were cut into 5 mm lengths and then incubated in Krebs-Henseleit buffer for 60 min at 37°C in a shaking water bath. After this period of time the tissue segments were incubated with 37 kBq ml\(^{-1}\) \[^3\]H-adenine (specific activity = 851 GBq mmol\(^{-1}\)) in Krebs-Henseleit buffer for a further 60 min at 37°C in a shaking water bath. Tissue segments were then washed three times with Krebs-Henseleit buffer before being transferred into flat-bottomed incubation vials (2 tissue segments per vial) containing Krebs-Henseleit buffer in a final volume of 300 \( \mu \)l. Each experiment was performed in quadruplicate. Vials were placed in a shaking water bath at 37°C and allowed to equilibrate for 10 min. All vials contained 0.1 \( \mu \)M U46619 and 1 mM isobutylmethylxanthine (IBMX). NPY (0.1 \( \mu \)M) or UK14304 (0.3 \( \mu \)M), or a combination of the two were added 10 min after the addition of IBMX and 5 min before the addition of forskolin reactions were terminated by addition of 200 ml of 1 M HCl. Vials were left on ice for 30 min, before the addition of 750 ml distilled water. One hundred micro litres of buffer was removed for total \[^{3}\]H counts.

\[^{3}\]H-cyclic AMP was separated from \[^{3}\]H-adenine and other \(^3\)H-products by alumina column chromatography. Briefly, 1 ml of the reaction buffer was added to 100 \( \mu \)l of \[^{14}\]C-cyclic AMP (30 Bq per tube) and applied to alumina columns. The eluate was discarded, and then the columns were washed twice with 4 ml of 5 mM HCl. One ml of 0.1 M ammonium acetate was then added to the columns and the eluate discarded. \[^{3}\]H-cyclic AMP was eluted from the columns with 3.5 ml of 0.1 M ammonium acetate. Levels of \[^{3}\]H-cyclic AMP and \[^{14}\]C-cyclic AMP in the eluate were measured by liquid scintillation counting. \[^{3}\]H-cyclic AMP levels were adjusted for the recovery from the alumina column chromatography (using the \[^{14}\]C-cyclic AMP as a standard) and also for the amount of total \(^{3}\)H taken up into the tissue.

**Drugs**

Neuropeptide Y (NPY) (Bachem (U.K.) Ltd.); (5Z, 9a, 11a, 13E, 15(S))-15-hydroxy-9 (11) methanoepoxyprosta-5,13-dienoic acid (U46619) (Cascade Biochem Ltd); 3-Isobutyl-1-methylxanthine (IBMX) (Sigma); Forskolin (Sigma); \[^{3}\]H-adenine (Amersham); \[^{14}\]C-cyclic AMP (NEN-DuPont); cumulative concentration-response curves to NPY (0.3 nM to 0.3 \( \mu \)M) were then constructed. Increases in the tone induced by NPY were measured from the pre-NPY baseline.
sodium nitroprusside (David Bull Labs); angiotensin II (Ciba); (R)-N₂-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine amide (BIBP 3226) (Dr Karl Thomae GmbH, Germany); 5 - bromo - 6 - [2 - imidazolin - 2-ylamine] - quinoxaline bitartrate (UK14304), (Pfizer); N⁶,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP) (Sigma), l-phenylephrine HCl (Sigma). All other compounds were obtained from Sigma, Pool, U.K.

Statistics
An F-test for equal variances was performed on all the data to test for normality. For single comparisons, normally distributed data were subjected to a Student’s two-tailed, unpaired t-test. Data which were not normally distributed were subjected to a non-parametric two-tailed, Mann-Whitney U-test. Multiple comparisons were performed using analysis of the variance (ANOVA) followed by a Bonferroni test.

Contractile responses were expressed as a percentage of the final response to 60 mM KCl. Results were expressed as mean ± s.e.mean. Statistical significance was assumed when P < 0.05.

Results
Effect of pre-contraction of the porcine isolated ear artery on NPY responses
NPY (0.1 μM) produced a small contraction in the porcine isolated ear artery equivalent to 5.1 ± 0.8% (mean ± s.e.mean; n = 14) of the response to 60 mM KCl (2.4 ± 0.2 g wt, n = 14). Angiotensin II (0.3 μM) produced a transient contraction (maximum 81.4 ± 10.6%, duration 10–15 min, n = 4) which returned to baseline. Subsequent addition of 0.1 μM NPY produced a small contraction of the tissue which was not significantly different from that seen in the absence of angiotensin II (10.5 ± 1.5% in the presence of angiotensin II compared to 9.9 ± 5.0% in the absence of angiotensin II; n = 4).

In a separate series of experiments, porcine isolated ear arteries were pre-contracted with the thromboxane-mimetic U46619 (0.1 μM). U46619 produced a sustained contraction (102.9 ± 13.4%, n = 4). The responses were allowed to reach a plateau before the addition of 0.1 μM NPY. NPY produced a further contraction of the tissue. However, the NPY response was not significantly different from that obtained in the absence of U46619 (6.5 ± 3.3% after pre-contraction with U46619 compared to 10.1 ± 4.1% in the absence of U46619; n = 4).

Effect of pre-contraction with U46619, and relaxation with forskolin on NPY responses
Typical traces showing the effect of 0.1 μM NPY in the presence or absence of 0.1 μM U46619 and forskolin on porcine isolated ear artery segments are shown in Figure 1. NPY concentration-response curves were constructed in the porcine ear artery in the presence or absence of the combination of U46619 and forskolin. In tissues which had been exposed to U46619 and forskolin prior to being washed with Krebs-Henseleit buffer, NPY produced a contraction at only the highest concentrations employed (Figure 2). However,
in tissues which had been pre-contracted with U46619, and relaxed with forskolin, NPY produced an enhanced response over most of the concentration-response curve (Figure 2). The NPY Y1-specific non-peptide antagonist BIBP 3226 (0.7 μM) inhibited this enhanced response, with NPY only producing a response at the highest concentration employed (Figure 2).

**Effect of NPY on forskolin-stimulated cyclic AMP levels**

Cyclic AMP levels were measured in conditions similar to those used in the functional studies (i.e. in the presence of 0.1 μM U46619). However, we have previously been unable to detect an increase in cyclic AMP levels in the presence of 1 μM forskolin (a concentration which is comparable to that used to relax the tissues in the functional studies) in the porcine isolated ear artery (Roberts et al., 1998). Therefore, cyclic AMP measurements were also performed in the presence of 1 mM IBMX to increase the cyclic AMP to detectable levels.

In the presence of IBMX and U46619, the basal value of [3H]-cyclic AMP in the porcine ear artery segments was 0.6±0.1% (n=9) (expressed as per cent conversion of [H] taken up into the tissue). Forskolin (1 μM) increased [3H]-cyclic AMP levels 7 fold (Figure 3). NPY (0.1 μM) reduced forskolin-stimulated cyclic AMP by 40%, but had no significant effect on basal levels of cyclic AMP (Figure 3).

**Effect of different relaxing agents on NPY responses**

Tissues were pre-contracted with 0.1 μM U46619 and relaxed back to baseline with either forskolin (1–2 μM), sodium nitroprusside (SNP; 100–200 μM), or dibutyryl cyclic AMP (3–5 mM). Tissues were then exposed to a single, submaximal concentration of NPY (0.1 μM). The vasoconstrictor response to NPY in the ear artery was enhanced after relaxation with forskolin, SNP, or dibutyryl cyclic AMP when compared with the responses to NPY in the absence of U46619 and relaxing agent (Figure 4). However, the responses to NPY after relaxation with forskolin (49.8±5.3%, n=14) were significantly greater than those seen after relaxation with SNP (28.9±5.7%, n=11), or dibutyryl cyclic AMP (21.2±4.6%, n=14) (ANOVA followed by a Bonferroni test). There was no significant difference between the responses to NPY after relaxation with either SNP or dibutyryl cyclic AMP.

**Effect of UK14304 on subsequent response to NPY**

Previous studies have demonstrated that enhanced responses to the a2-adrenoceptor agonist UK14304 can also be obtained after pre-contraction with U46619 and relaxation with...
forskolin (Roberts et al., 1998). In a separate set of experiments, forskolin-stimulated cyclic AMP (4.5 ± 0.3% conversion) was significantly reduced to a similar degree by 0.1 µM NPY (3.3 ± 0.2% conversion, n = 8) and 0.3 µM UK14304 (3.2 ± 0.2% conversion, n = 8) (both ANOVA followed by a Bonferroni test). However, a combination of 0.1 µM NPY and 0.3 µM UK14304 failed to significantly reduce forskolin-stimulated cyclic AMP production any further (2.8 ± 0.2% conversion, n = 8).

As the effects of 0.3 µM UK14304 and 0.1 µM NPY on the inhibition of forskolin-stimulated cyclic AMP levels were not additive, it was of interest to determine whether this was also the case with the contractile response. Tissues were precontracted with 0.1 µM U46619, and relaxed back to baseline with forskolin prior to addition of 0.3 µM UK14304. Under these conditions, 0.3 µM UK14304 produced a contraction which was 58.2 ± 2.7%, n = 8. Although subsequent addition of 0.1 µM NPY produced a further contraction of the tissue, the size of the NPY response was similar to that seen with 0.1 µM NPY alone (6.9 ± 1.9%, after UK14304, n = 8, compared to 7.2 ± 3.2, NPY alone n = 7).

**Discussion**

NPY produces direct vasoconstrictor responses in a few isolated vessels including cerebral arteries from the cat (Fredholm et al., 1985), rat (Xia et al., 1992), and rabbit (Abel & Han, 1989), and coronary arteries from rabbit (Han & Abel, 1987). As shown in the present study, NPY also produces a small vasoconstrictor response in the porcine isolated ear artery. While previous reports have illustrated that pre-contraction of blood vessels with agents such as endothelin, phenylephrine, or 5-HT can enhance contractions produced by NPY (Abel & Han, 1989), and coronary arteries from rabbit (Han & Abel, 1987), the contractile responses to NPY in the porcine isolated ear artery are mediated via a different mechanism similar to that observed for 2-adrenoceptor-mediated vasoconstriction in the rat femoral artery (Fredholm et al., 1991), although the magnitude of the response was only half of that obtained in the presence of forskolin (Roberts et al., 1998).

The contractile responses to NPY and UK14304 after relaxation with forskolin could be explained by the ability of the agents to reduce cyclic AMP levels, thereby permitting the U46619 contractile response to return. However, as we have previously reported, a second intracellular pathway appears to be present in the porcine ear artery by which 2-adrenoceptor agonists can produce an enhanced response (Roberts et al., 1998). UK14304 alone is able to produce a small contraction in the porcine isolated ear artery without reducing basal levels of cyclic AMP (Roberts et al., 1998). Furthermore, enhancement of responses to UK14304 were also noted in the porcine ear artery after the tissue had been relaxed with dibutyryl cyclic AMP, a cell permeable, protein kinase A activator (Hei et al., 1991), although the magnitude of the response was only half of that observed in the presence of forskolin (Roberts et al., 1998). These data suggest that at least part of the 2-adrenoceptor-mediated response is due to an action distal to the changes in cyclic AMP i.e. adenylyl cyclase-independent. In the present study NPY alone also elicited a small contraction in the porcine ear artery without reducing basal levels of cyclic AMP, and produced a small contraction in the presence of raised cyclic AMP levels (in the presence of UK14304 after pre-contraction with U46619 and relaxation with forskolin). Furthermore, NPY elicited an enhanced contraction following relaxation with either dibutyryl cyclic AMP, or SNP (which also produces a relaxation of the ear artery independent of changes in cyclic AMP (Roberts et al., 1998)). The responses to NPY after relaxation with dibutyryl cyclic AMP or SNP were similar to those obtained with UK14304 under identical conditions in that they were greater than the contraction to NPY alone, but less than the contraction after relaxation with forskolin. These results indicate that NPY can produce a contraction of the porcine ear artery via an adenylyl cyclase-independent mechanism similar to that observed for 2-adrenoceptors. In the rat femoral artery, an enhanced NPY-mediated contraction can be obtained by pre-contraction with phenylephrine and relaxation with sodium nitroprusside or subsequent relaxation with forskolin, SNP, or dibutylryl cyclic AMP (Roberts et al., 1998).

Both NPY Y1 receptors and 2-adrenoceptors are considered to be negatively-coupled to adenylyl cyclase (Balasubramaniam, 1997; Bylund et al., 1994). Under similar conditions to those used in the contractile studies, NPY (this study) and UK14304 (this study and Roberts et al., 1998) were able to inhibit forskolin-stimulated cyclic AMP formation to a similar degree, although the responses were not additive. The combination of NPY and UK14304 did not significantly reduce cyclic AMP levels any further than the reduction seen with either agent alone, indicating that the two compounds inhibit cyclic AMP formation through different pathways. Tissues which had been stimulated with UK14304 after pre-contraction with U46619 and relaxation with forskolin, subsequent addition of NPY (on top of the UK14304 response) produced a further contraction, comparable to that seen with NPY alone. In other words, the contractile responses to NPY and UK14304 in the presence of U46619 and forskolin are not additive, and this is mirrored by the effects observed on cyclic AMP levels. Again, this provides further evidence that the enhanced responses to NPY and UK14304 under these conditions are mediated through the same pathway. It is also interesting to note that, while UK14304 reduced cyclic AMP levels by approximately 30%, the levels of cyclic AMP were still approximately 5 fold higher than basal values. In spite of the prevailing inhibitory influence of cyclic AMP, NPY was still able to elicit a small contraction of the porcine ear artery – evidence for an adenylyl cyclase independent pathway.
histamine (Grundemar & Hogestatt, 1992). This demonstrates that similar pharmacological manipulation in different tissues may produce a similar enhancement of responses to NPY.

A recent study in rat mesenteric small arteries also supports the view that NPY can elicit contractions through two independent pathways, although the study did not examine NPY responses after pre-contraction with another agonist (Prieto et al., 1997). The first pathway involves the ability of NPY to inhibit forskolin-stimulated cyclic AMP formation, and, therefore, prevents the effects of cyclic AMP accumulation on membrane potential. The second pathway involves a direct depolarization of the arterial smooth muscle through activation of gadolinium-sensitive cation channels (Prieto et al., 1997). NPY is also able to increase [Ca\(^{2+}\)]\(_i\) in an adenylyl cyclase-independent manner (Erdrbrugger et al., 1993; Mihara et al., 1989), although this is thought to be mediated through a Y\(_2\)-like receptor (Feth et al., 1991). As an increase in [Ca\(^{2+}\)]\(_i\), leading to an increase in myosin light chain kinase activation is generally considered to be the primary cause of vasoconstriction (Rembold, 1992), it is possible that the contraction to NPY alone in the porcine ear artery is the result of an increase in [Ca\(^{2+}\)]\(_i\) through an adenylyl cyclase-independent pathway. In the presence of U46619/dibutyryl cyclic AMP or SNP this increase in [Ca\(^{2+}\)]\(_i\), may be enhanced. Alternatively, the adenylyl cyclase-independent mechanism could involve sensitization of the contractile proteins to calcium, such that NPY is able to produce a contraction with little change in [Ca\(^{2+}\)]\(_i\). Calcium sensitization accounts for part of the \(\alpha_2\)-adrenoceptor response in the rabbit saphenous vein (Aburto et al., 1993) and has been advanced as a mechanism underlying 5-HT\(_1\)-like contractions of the rabbit isolated femoral artery (Randall et al., 1996). Interestingly, in the latter study 5-HT was shown to inhibit forskolin-stimulated cyclic AMP, yet pharmacological manipulation with a vasoconstrictor (angiotensin II) and an adenylyl cyclase-independent relaxant (SNP) was also found to enhance 5-HT\(_1\)-like vasosconstriction. Taken together, the above findings highlight the need for further studies on the adenylyl cyclase-independent vasoconstrictor mechanisms associated with these receptors.

In conclusion, we have demonstrated that an enhanced contraction to NPY in the porcine isolated ear artery can be obtained by prior pharmacological manipulation. Biochemical examination of the response suggests the involvement of adenylyl cyclase-dependent and independent pathways, qualitatively similar to those reported for \(\alpha_2\)-adrenoceptors in this preparation (Roberts et al., 1998).

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**References**


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