BIOCHEMICAL LETTERS

Limitations of the radioreceptor assay of inositol 1,4,5-trisphosphate in vanadate-treated cell suspensions

Recent studies have shown that the insulinomimetic agent vanadate or pervanadate evokes a significant increase in the levels of inositol 1,4,5-trisphosphate (IP_3) in various cell types. Some of these investigations have utilized a rapid and sensitive radioreceptor assay which involves competitive inhibition of $[^3H]IP_3$ specific binding to membrane preparations rich in IP_3 target sites. By this methodology, a time-dependent rise in IP_3 levels has been observed in vanadate-treated HL60 granulocytes (Bianchin et al., 1993) and rat adipocytes (Morita et al., 1992). The mechanism underlying the elevation of IP_3 by vanadate has not been clearly described, although activation of phospholipase C γ (Bianchin et al., 1993; Randazzo et al., 1992) and inhibition of inositol phosphatase activity (Bencherif and Lucas, 1992) have been proposed. However, the present study confirms that the observed increase in IP_3 measured by the radioreceptor method is an artefact, since acidification/neutralization and/or extraction procedures result in the formation of decavanadate, which itself is a potent inhibitor of IP_3 binding to its receptor (Fohr et al., 1991).

Aortic smooth-muscle cells (ASMC) isolated from adult male Sprague-Dawley rats and cultured as described previously (Gopalakrishnan et al., 1991), were treated with increasing concentrations of vanadate [prepared as described by Kadota et al. (1987a) in Krebs-Ringer bicarbonate Hepes (KRBH) buffer, final pH 7.4] at 37 °C for 10 min. The reaction was terminated by using 20% $HClO_4$. Control KRBH buffer (containing no ASMC), was also subjected to treatments with similar concentrations of vanadate and $HClO_4$. The acidified extracts were centrifuged and the supernatant was neutralized with 1.5 M KOH. Alternatively, vanadate (1 mM)-treated (10 min at 37 °C) KRBH buffer, pH 7.4, was acidified with 20% trichloroacetic acid (TCA), subjected to centrifugation, and the resultant supernatant was extracted with 3 × 1 ml of water-saturated diethyl ether. The samples from both the procedures were allowed to compete with 1.2 nM $[^3H]IP_3$ in the presence of bovine adrenocortical microsomes (BACM). The composition of the binding-assay mixture, incubation and quantification methods for IP_3 were the same as described previously (Palmer and Wakelam, 1990; Challiss et al., 1990).

The pattern of inhibition curves and the calculated final IC_{50} values were closely similar for both the control (KRBH buffer) group ($174 \pm 9 \mu M$) and the ASMC group ($172 \pm 12 \mu M$), as shown in Figures 1(a) and 1(b). Moreover, an orange-yellow colour was formed in the incubation mixture upon acidification and alkali treatment, confirming the formation of a polymeric condensation product, possibly decavanadate (Fohr et al., 1989). When an alternative procedure involving TCA treatment and ether extraction was used for the vanadate-treated KRBH buffer system, the degree of inhibition of $[^3H]IP_3$ binding was about

$68 \pm 4\%$, as against $44 \pm 2\%$ inhibition for $HClO_4/KOH$ treatments.

Previously, it has been demonstrated that decavanadate is formed on acidification of the monomeric and oligomeric forms of vanadate, and that decavanadate (IC_{50} 0.5 μM) inhibits specific binding of $[^3H]IP_3$ to BACM (Fohr et al., 1989, 1991). Since acid treatment (TCA or $HClO_4$) is commonly used in radioreceptor assays of IP_3 in order to arrest the reaction, we investigated the possible interference of this procedure on $[^3H]IP_3$ binding by the resultant condensation products of vanadate. Our data show

Figure 1 Competitive inhibition of increasing concentrations of vanadate (1 μM –5 mM) in KRBH (a) buffer (250 μl , pH 7.4) or rat ASMC (b) suspension (10^6 cells in 250 μl of KRBH buffer, pH 7.4) on $[^3H]IP_3$ (1.2 nM) specific binding to BACM

The inhibition curves for (a) and (b) were superimposable, with closely similar IC_{50} values. Each data point shown is the mean of four separate experiments, each performed in either duplicate or triplicate; S.E.M. values were less than 5% for each data point.

that vanadate treatment does not cause an increase in IP_3 content, at least by the radioreceptor method, as evidenced by similar IC_{50} values and displacement curves in the competition assays for both the ASMC group and the control group that received KRBH buffer alone.

The contradictory observations of Lonnroth et al. (1993) on the effects of vanadate species in human adipocytes, as against those observed by Kadota et al. (1987b) in rat adipocytes, may be easily explained by the fact that 1 M HCl (which strongly favours formation of decavanadate) had been used by Lonnroth et al. (1993) to adjust the pH of a 10 M vanadate solution to 7.4. The observed rapid and massive elevations of IP_3 by the radioreceptor method after treatment of fat-pads with 2 mM vanadate (Morita et al., 1992) and of granulocytic HL60 cells with 100 μ M vanadyl peroxide (Bianchin et al., 1993) may be apparent as the formation of decavanadate is unavoidable by both procedures.

It is concluded that the radioreceptor assay may not give a true index of changes in IP_3 levels after treatment of cell preparations with vanadate at higher concentrations, as there is a possibility of interference due to oligomeric and decameric (predominantly) vanadate species.

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