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HEMOGLOBIN CAUSES RELEASE OF INOSITOL TRISPHOSPHATE FROM VASCULAR SMOOTH MUSCLE

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To test the hypothesis that oxyhemoglobin causes contraction of vascular smooth muscle by production of inositol 1,4,5-trisphosphate which results in a release of intracellular calcium, smooth muscle cells were exposed to oxyhemoglobin and inositol trisphosphate was measured. Oxyhemoglobin, but not methemoglobin which has much less contractile action, stimulated inositol trisphosphate production. The time course was consistent with an early role for this compound in the contraction produced by hemoglobin. The increase in production of inositol trisphosphate was inhibited by pertussis toxin and also by neomycin, an inhibitor of phospholipase C, although the actions of the latter compound cannot be attributed only to an inhibition of the enzyme responsible for the production of inositol trisphosphate.

OxyHb produces a contraction of cerebral and peripheral blood vessels and there is substantial evidence that the release of this compound from lysed erythrocytes in the subarachnoid clot may be responsible for the cerebral vasospasm that occurs after subarachnoid haemorrhage (1). However, the cellular mechanism by which OxyHb causes contraction is still not clear. The greater contractile activity of ferrous proteins such as OxyHb, as compared with ferric proteins such as MetHb, suggests that superoxide radicals produced by auto-oxidation of OxyHb to MetHb may be a cause of the vasoconstriction. However, scavengers of free radicals had no effect on the ability of OxyHb to produce contraction of isolated blood vessels (2). The removal of the vascular endothelium does not affect the vasoconstrictor responses to OxyHb (3), and thus it seems unlikely that changes in the release of endothelium-derived relaxing or constricting substances is the primary cause of vasospasm. One possible mechanism by which OxyHb-induced vasoconstriction might be mediated involves the opening of voltage-operated calcium channels, but since neither the response to OxyHb nor the development of cerebral vasospasm after subarachnoid hemorrhage are antagonized well by calcium channel antagonists (4) it is more probable that at least the early contraction to OxyHb results from the release of intracellular Ca^{2+}. The observation that intracellular Ca^{2+} antagonists, such as HA1077, may prevent cerebral vasospasm (5) is consistent with this suggestion. Vascular smooth muscle cells are known to respond to norepinephine, thrombin and angiotensin II by rapid hydrolysis of membrane inositol phospholipids, thus generating two second messengers, Ins(1,4,5)P_3, and 1,2-diacylglycerol, which produce an increase in the intracellular Ca^{2+} levels and an activation of protein kinase C, respectively

Abbreviations: OxyHb = oxyhemoglobin, MetHb = methemoglobin, Ins(1,4,5)P_3 = inositol (1,4,5)trisphosphate, Ins(1,3,4,5)P_4 = inositol (1,3,4,5)tetrakisphosphate.

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(6). We report here that an early signalling event in the contraction produced by OxyHb in vascular smooth muscle may involve formation of Ins(1,4,5)P_3.

MATERIALS AND METHODS

Myo[^{3}H]inositol was obtained from American Radiolabelled Chemicals (St. Louis, MD). [^{3}H]Ins(1,3,4,5)P_4, a [^{3}H]inositol polyphosphate marker set which contains [^{3}H]inositol phosphate, [^{3}H]inositol 1,4-bisphosphate and [^{3}H]Ins(1,4,5)P_3, and an Ins(1,4,5)P_3 binding assay system were all purchased from Amersham Radiochemicals. OxyHb and MetHb were prepared from human hemoglobin (Sigma) by the method of Martin et. al. (7). The concentrations of both hemoproteins was determined by ultraviolet absorption spectrophotometry (8). Other chemicals were commercial products of the highest grade available.

Rat aortic smooth muscle cells (A_7r_5) were purchased from the American Type Tissue Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were grown at 37°C. in an atmosphere containing 5% carbon dioxide and were passaged weekly.

Cells were incubated for 72 hours in DMEM containing myo[^{3}H]inositol (20μCi/ml). Labelled cells were detached with 0.01% trypsin, washed three times with DMEM and pre-incubated at 37°C. for 10 minutes in a medium which contained Hepes tris buffer (pH 7.4), NaCl (120 mM), KCl (5mM), $MgCl_2$ (1mM), $CaCl_2$ (1.5mM), K_2HPO_4 (1mM), glucose (5.5mM) and LiCl (10mM). The cells were then exposed to OxyHb or MetHb for the appropriate time and the reaction was terminated by addition of ice-cold trichloracetic acid (15%). The suspension was kept on ice for 20 minutes and centrifuged at 20,000 x g for 15 minutes at 4°C. The supernatant was extracted three times with ten volumes of diethylether saturated with water, and brought to pH 7.5 with sodium bicarbonate. The inositol polyphosphates were separated using HPLC as described previously (9). The solvent was ammonium formate (1.7 M) buffered to pH 3.7 with phosphoric acid and the program was water (5 min) followed by a linear increase to 100% solvent (50 min). Each collected fraction had a volume of 0.5ml (1-14 min and 38-55 min) or 0.2ml (14-38 min). The peaks were identified by comparison with standard inositol polyphosphates and also with AMP, ADP and ATP.

Ins(1,4,5)P_3 was measured in supernatant fractions from A_7r_5 cells and in slices of rat aorta using a specific Ins(1,4,5)P_3 binding protein (Amersham). The mixture was incubated at 0°C. and centrifuged at 2000 x g for 10 min at 4°C. Radioactivity was determined using a Beckmann LS6800 scintillation counter and the amount of Ins(1,4,5)P_3 was estimated from a standard curve.

Contractility studies were conducted using rings of rat aorta suspended under a resting tension of 1 g. in standard organ baths of 10 ml working volume. The baths were maintained at 37°C. and contained Krebs-Henseleit solution (10) gassed with 95% oxygen:5% carbon dioxide. Contractions were recorded isometrically using a Grass model 7d polygraph connected to Grass FT.03 strain gauges. The tissues were allowed to equilibrate for one hour before drugs were applied.

Statistical significance was determined by Student's 't'-test for unpaired data or analysis of variance as appropriate.

RESULTS

Formation of Inositol polyphosphates in cultured aortic cells Incubation of confluent A_7r_5 cells with 10 μM OxyHb resulted in a rapid elevation in the levels of inositol phosphates. HPLC analysis of extracts from control and OxyHb-stimulated cells showed that both the 1,4,5- and the 1,3,4- isomers of $InsP_3$ were present shortly after stimulation with OxyHb (Fig 1), the former being elevated by 116 and 180% of control after 30 and 60 sec, respectively. An increase of about 190% in Ins(1,3,4,5)P_4 was seen after 60 sec stimulation, and this is likely to arise from phosphorylation of Ins(1,4,5)P_3 (11). Unstimulated preparations show only trace amount of Ins(1,3,4,5)P_4. A modest increase in the amount of Ins(1,4)P_2 is present at 30 and 60 sec which is presumably derived from the action of a phosphomonoesterase on Ins(1,4,5)P_3 (12).

Formation of Ins(1,4,5)P_3 in A_7r_5 cells stimulated with OxyHb, MetHb and other vasoconstrictors The [^{3}H]-Ins(1,4,5)P_3 assay system contains a specific (1,4,5)InsP_3 binding protein; using this assay it is possible to obtain an accurate estimate of the concentration of the 1,4,5-isomer, even in the

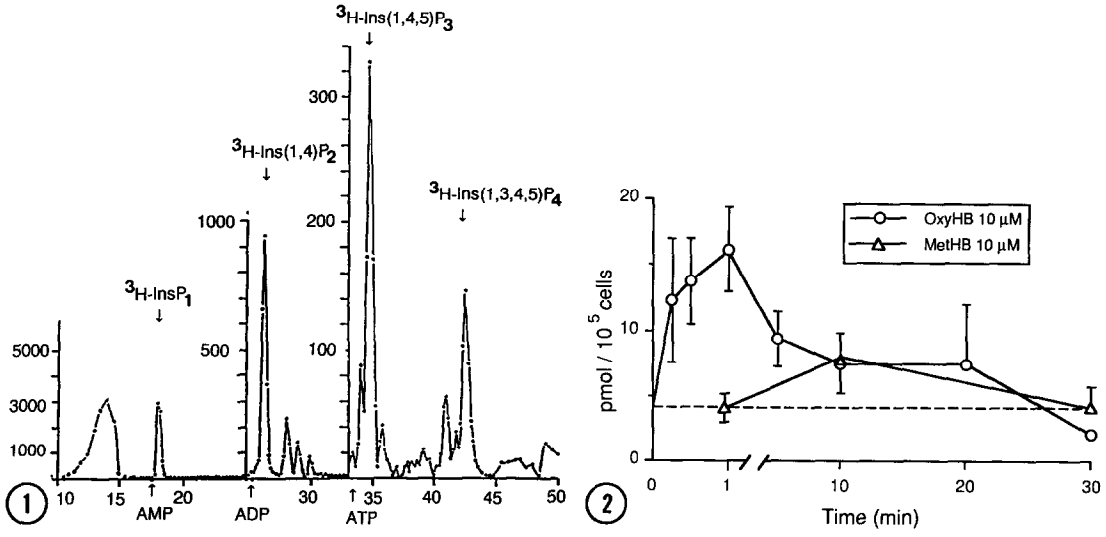

Fig. 1. Counts/min from HPLC fractions after stimulation of A_7r_5 cells with OxyHb (10 μM) for 60 sec. Arrows show retention of standard marker compounds.

Fig. 2. $Ins(1,4,5)P_3$ generated after treatment of A_7r_5 cells with OxyHb and MetHb (both 10 μM) as a function of time.

presence of substantial amounts of $Ins(1,3,4)P_3$. OxyHb(10μM) stimulated the production of $Ins(1,4,5)P_3$ as early as 15 sec after exposure, with a maximal effect at one minute (Fig 2). However, the finding that after about 5 min, the release of $Ins(1,4,5)P_3$ is not significantly different from control values, suggests that the effects of OxyHb on the formation $Ins(1,4,5)P_3$ are relatively transient. In contrast to the findings with OxyHb, treatment with MetHb at the same concentration did not result in a significant increase in $Ins(1,4,5)P_3$ at any time. As a positive control, the effects of norepinephrine (10μM) and thrombin (10 U/ml) were also examined in A_7r_5 cells. Both produced a highly significant elevation of $Ins(1,4,5)P_3$ at 60 sec as shown in Fig 3. The observation that OxyHb but not MetHb elevate $Ins(1,4,5)P_3$ was confirmed using tissue slices from rat aorta and the [^{3}H]-$InsP_3$ assay system (results

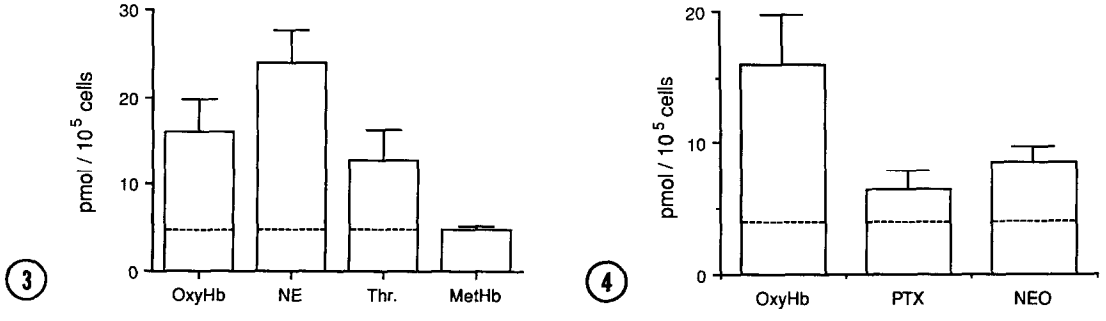

Fig. 3. $Ins(1,4,5)P_3$ generated after exposure for 60 sec to OxyHb (10 μM), MetHb (10μM), Norepinephrine (NE, 10 μM) and Thrombin (Thr, 10 Units/ml). Bars indicate standard errors, dotted line represents basal level.

Fig. 4. Effects of pre-exposure to pertussis toxin (PTX, 400 ng/ml for 4 hr) and neomycin (NEO, 5 mM for 10 min) on the elevation of $Ins(1,4,5)P_3$ produced by exposure for 60 sec to OxyHb (10 μM). Bars represent standard errors, dotted line represents basal level.

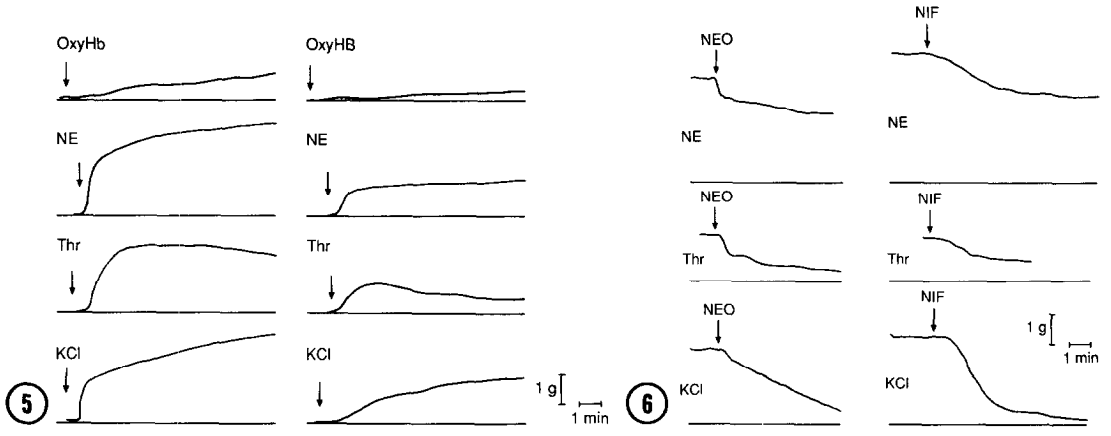

Fig. 5. Responses of ring preparations of rat aorta to OxyHb (10 μM), norepinephrine (NE, 10 μM) and thrombin (Thr, 10 Units/ml), before (left panel) and after (right panel) exposure to neomycin (5 mM) for 10 min.

Fig. 6. Effects of Neomycin (NEO, 5mM) and Nifedipine (NIF, 1 μM)) on contractions produced in rat aortic ring preparations by norepinephrine (NE, 10 μM), thrombin (Thr, 10 units/ml) and potassium chloride (KCl, 50 mM).

not shown). MetHb, even at a concentration of 100 μM, produced a maximum increase of only about 141% in Ins(1,4,5)P_3 as compared to 287% produced by OxyHb at the same concentration.

In A_7R_5 cells which had been preincubated with pertussis toxin (400 ng/ml) for 4 hr before stimulation with OxyHb(10μM), the release of Ins(1,4,5)P_3 was not significantly different from that found in unstimulated cells, suggesting that OxyHb stimulates phospholipase C via a pertussis toxin-sensitive G protein (Fig 4). Neomycin is reported to inhibit phospholipase C directly (13,14), and at a concentration of 5mM this agent did produce a significant decrease in the amount of Ins(1,4,5)P_3 generated by OxyHb. These data are also shown in Fig 4.

Contractile effects of OxyHb on rat aortic rings In order to examine the effects of agents which may alter the possible signal transduction mechanisms for the OxyHb-induced contraction, the ability of such agents to affect the contraction was studied using standard organ-bath techniques. Because OxyHb produces a contraction which develops slowly and is reversible only after very prolonged wash, the preparations were exposed to OxyHb at a single concentration (10μM) and the response was recorded for at least 20 min. The effects are shown in comparison with those of norepinephrine, thrombin and potassium chloride in Fig 5. The inhibition of the response by prior exposure to neomycin (5mM) for 10 min is also shown in Fig 5. The response to each agent is diminished, and the onset of the response is delayed in each case. When neomycin or the calcium antagonist nifedipine (10μM) were administered to preparations in which a tonic contraction to the four agonists had developed, it was found that thrombin and norepinephrine were relaxed somewhat while the effects of potassium chloride were all but abolished. Under the same conditions the response to OxyHb was only minimally affected by either agent (Fig 6).

DISCUSSION

For the examination of the effects of OxyHb on signal transduction mechanisms, we chose to use an established cell line from rat aorta. While the clinical significance of the effects of OxyHb are

most important in the cerebral vasculature, a number of workers have used preparations obtained from peripheral vessels to examine the response. In particular Fuji and Fujitsu (15) showed that OxyHb produced changes in isolated rat aortic smooth muscle cells which closely resembled those seen in cerebral vessels after intracranial hemorrhage.

It is clear from the data discussed above that OxyHb can stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate with a consequent elevation of Ins(1,4,5)P_3. It is well established that an increase in intracellular Ins(1,4,5)P_3 can act as a second messenger to promote release of calcium from sarcoplasmic reticulum which, in turn activates the contractile machinery of the cell (16). This has been shown to be the mechanism by which a variety of agonists produce contraction in vascular smooth muscle as well as smooth muscle from other preparations (17). OxyHb produces its maximum effects on the production of Ins(1,4,5)P_3 after about one minute. This precedes the contraction which is produced by OxyHb, which is consistent with a second messenger role for Ins(1,4,5)P_3 (18). The responses to norepinephrine and thrombin occur much more rapidly than that to hemoglobin, but evidence from other laboratories suggests that the elevation in the formation of Ins(1,4,5)P_3 by these agents also occurs more rapidly (19). The increase in formation of this second messenger by OxyHb is brief; within about ten minutes the levels have returned almost to normal, although the contraction is sustained for much longer time periods. MetHb is largely devoid of contractile effects and, as expected also lacked an effect on the levels of Ins(1,4,5)P_3. This observation also supports the idea that Ins(1,4,5)P_3 plays a second messenger role in the OxyHb-induced contraction. The early elevation of Ins(1,4,5)P_3 is sensitive to pertussis toxin. At present it seems likely that there are at least two different G-proteins which are coupled to phospholipase C, one of which is sensitive to pertussis toxin while the other is not (20). Responses of vascular smooth muscle have variously been reported to be either sensitive or insensitive to pertussis toxin, depending on the agonist and on the blood vessel involved (21-24). It is not yet clear whether OxyHb can stimulate the coupling of the G-protein to phospholipase C directly, or whether it promotes release of some other compound or compounds which in turn stimulate the G-protein and thus the phospholipase C.

Neomycin has been reported to be an antagonist of phospholipase C, and extracellular application of this agent inhibits phospholipase C in a variety of preparations including smooth muscle (13,14,25). Preincubation with neomycin at a concentration of 5mM reduced the OxyHb-induced elevation in Ins (1,4,5)P_3 by more than 50%, and this is consistent with the proposed mechanism of action on phospholipase C. Neomycin also delayed and diminshed the pharmacological responses to OxyHb, norepinephrine, thrombin and potassium chloride and while this is consistent with an inhibition of the production of the second messenger, the data with potassium chloride show that this agent may have some additional actions, since potassium chloride is generally believed to produce contraction by promoting calcium entry through dihydropyridine-sensitive calcium channels. While there is some suggestion that the process of calcium entry may be coupled in some ways with levels of intracellular inositol polyphosphates, particularly Ins(1,3,4,5)P_4(26), it would not be expected that the response to potassium would be attenuated by inhibition of phospholipase C to a similar extent to those agents which depend on Ins(1,4,5)P_3 to release calcium from inside the cell.

The tonic response to all agonists tested is unlikely to arise from a continuing elevation of Ins (1,4,5)P_3. After twenty minutes the second messenger levels have returned to control levels even though the contraction is sustained. Neomycin also causes relaxation under these circumstances and

the close similarity between the effects of this compound and those of nifedipine suggest the possibility that it is possible that neomycin is affecting a component of calcium influx. Neither neomycin nor nifedipine have much effect on the response to OxyHb.

The results suggest that the response to OxyHb is initially mediated by an increase in $Ins(1,4,5)P_3$ generated by activation of phospholipase C through a pertussis toxin-sensitive G-protein. The prolonged response to oxyhemoglobin is not mediated directly by phospholipase C, and does not appear to be the result of influx of calcium through dihydropyridine-sensitive channels. Probably, OxyHb promotes calcium entry by some other mechanism which may be triggered by the initial elevation in inositol polyphosphates.

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