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MECHANISM OF ACTION OF HYDRALAZINE ON VASCULAR SMOOTH MUSCLE

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Abstract—Myofibrils prepared from bovine carotid arteries were used to investigate the hypotensive action of hydralazine. These myofibrils contained an ATPase and Protein Kinase which was half-maximally activated by 1 μM Ca^{2+}. Hydralazine inhibited Ca^{2+} dependent ATPase and phosphorylation. Half maximal inhibition occurred at 2×10^{-5} M hydralazine. This inhibition was accounted for by a specific reduction in the phosphorylation of a protein which migrated with the myosin P-light chains (M_r, 20,000). Phosphorylation of the latter protein is generally thought to be obligatory for muscle contraction. Thus an inhibition of this phosphorylation by hydralazine *in vivo* is likely to contribute to the hypotensive action of the drug.

The hypotensive drug, hydralazine, inhibits the stimulant action of many different agonists on vascular and visceral smooth muscle [1]. This non-selective action is indicative of a direct effect on smooth muscle as opposed to an antagonism of a transmitter at a specific cell receptor.

The mechanism of the hypotensive effect of hydralazine in producing vasodilation both *in vivo* and on contracted arterial strips is uncertain. The scant evidence that exists suggests an interference with the influx of Ca^{2+} into the smooth muscle cell or its release from intracellular stores [2]. The relationship, however, between Ca^{2+} influx and contraction is not understood but is certainly mediated by complex biochemical pathways. It is conceivable that a decrease in Ca^{2+} influx is secondary to other events related to the regulation of contraction. Similarly, the increase in cyclic nucleotide concentration often found associated with the relaxant effect on smooth muscle by vasodilators including hydralazine [3], may not have a direct causal relationship with vascular relaxation [4].

Contraction of vascular smooth muscle is produced by the interaction of actin and myosin [5]. Another possibility for the site of action of hydralazine is therefore the contractile apparatus itself and in particular the actomyosin. The activation of the interaction between actin and myosin is controlled through the myosin P-light chains [6, 7]. The extent of this interaction is determined by the concentration of Ca^{2+} ions within the sarcoplasm. Thus a rise in Ca^{2+} concentration regulates the actin activated myosin ATPase. Several biochemical steps have been identified in this regulatory pathway. Firstly, a rise in Ca^{2+} above about 10^{-7} M causes the formation of a Ca^{2+}-calmodulin complex, which in turn binds to the catalytic subunit of the MLCK*. MLCK then phosphorylates the myosin P light chains, which activates the actomyosin ATPase, cross-bridge formation and consequently muscle contraction [6, 7]. Relaxation is envisaged as resulting from a rapid dephosphorylation by a phosphatase possibility followed by a slower detachment of non-phosphorylated cross-bridges (latch).

It seems premature to consider other sites of action of hydralazine until its effect on arterial actomyosin is excluded. In this study, I have therefore investigated the action of hydralazine on the phosphorylation of myofibril protein and its actomyosin ATPase. My findings demonstrate that hydralazine inhibits the phosphorylation of the myosin P-light chains and the subsequent activation of the actomyosin ATPase. A direct action of hydralazine on the contractile apparatus of blood vessels may therefore contribute to the hypotensive effect of the drug.

EXPERIMENTAL PROCEDURES

Preparation of arterial myofibrils. Bovine carotid arteries were obtained at the time of slaughter, rapidly dissected free of fat and connective tissue and stored in Krebs Henseleit solution (Composition mM; NaCl 118, KCl 4.4, NaH_2PO_4 25, $MgCl_2$ 1.25, $CaCl_2$ 5.4, glucose 5.5). All subsequent procedures were performed at 4°. The adventitia was dissected and intima mechanically stripped away and discarded leaving strips of relatively pure muscularis.

Myofibrils were prepared by the method of Sobieszek and Bremel [8]. The muscularis was minced with scissors and placed in approximately 5 volumes of washing solution (60 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol 20 mM Imidazole adjusted to pH 6.9 containing 0.5% Triton). The tissue was then homogenized in a Brinkmann Polytron homogenizer for two bursts of 60 sec. The muscle residue was collected by centrifugation at 12,000 *g* for 10 min. This homogenizing and washing procedure was repeated three times. Then two washes were performed in the absence of the non-

* Abbreviations: K, kilodaltons; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; PSS, physiological salt solution; SDS, sodium dodecyl phosphate.

ionic detergent, Triton. Finally the tissue was washed and resuspended in PKM buffer (50 mM PIPES, pH 6.8, 60 mM KCl, 1 mM $MgCl_2$).

Measurement of phosphorylation and ATPase activity. Myofibrils were incubated at 37° in PKM buffer containing various concentrations of $CaCl_2$, EGTA and various concentrations of hydralazine (1-hydrazino phthalazine hydrochloride, Ciba Ltd., Horsham, Sussex, U.K.). Free Ca^{2+} concentrations were calculated using the data of [9]. The reaction was started by the addition of $\{\gamma - {}^{32}P\}$ ATP (5.1 Ci/mmol, Amersham International Ltd., Amersham, Bucks., U.K.) to a final concentration of 1 mM so as to give 5×10^5–10^6 cpm in 10 μl. Samples for ATPases measurements were taken at zero time and 10 min. The concentrations of protein was adjusted to give linear kinetics and approximately 50% hydrolysis of $\{\gamma - {}^{32}P\}$ ATP over the incubation time. Aliquots (10 μl) were removed from the incubation mixture and quenched in 1 ml 5% (v/v) perchloric acid containing 2% sodium pyrophosphate and 10 mg/ml Norit GSX charcoal. The charcoal was removed by centrifugation and 0.5 ml aliquots of the supernatant were assayed for radioactivity in 10 ml H_2O by Cerenkov radiation.

Phosphorylation of myofibril proteins was measured after washing the perchloric acid precipitated protein three times in 5% (w/v) trichloroacetic acid. Precipitates were then washed with ethanol and dissolved in SDS buffer (10 mM Tris, 1% (w/v) sodium dodecyl sulphate–1% (w/v) mercaptoethanol, pH 6.8). SDS was removed from samples before counting by the method of Zaman and Verwilghen [10]. Aliquots of these SDS-free samples were also assayed for protein. Phosphorylated proteins were analysed by SDS–PAGE (5–15% acrylamide gradient) using the buffer system of Laemelli [11]. The intensity of the radioactive bands were visualized by autoradiography and quantitated by densitometry using a Joyce Loebl Chromoscan. Autoradiography was performed for 2–4 days using Du Pont lightening plus intensifying screen and Kodak X-Omat RP X-ray film. The phosphate incorporation into the 20 K myosin P-light chains was proportional to the protein loaded onto the gel. The position of the myosin P-light chains was established using purified porcine aortic smooth muscle myosin and actomyosin (a generous gift from S. Marston, Cardiothoracic Institute, London).

Phosphate incorporation into intact bovine carotid artery strips. Vessels of approximately 5 cm in length and 0.5 cm in diameter were cut helically and mounted in two 30 ml jacketed water baths containing physiological salt solution (131 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.026 mM Na_2EDTA, 5 mM glucose, 30 mM Tris–Cl, pH 7.25) at 37° with constant oxygenation. Tension was measured with the aid of a Grass force transducer and a Grass model 5 polygraph. Two parallel strips were set up at a resting tension of 30 g and equilibrated for 90 min prior to experimental treatment. The kinetics of tension production were established by the addition of 10^{-6} M noradrenaline to each organ bath. The tissues were washed until the base line was restored to the original resting tension. With this protocol, further additions of noradrenaline gave reproducible contractions. Both strips were then preincubated with 1 mCi $\{^{32}P\}$ carrier free phosphate for 1 hr to equilibrate the intracellular phosphate pool. Before addition of drugs, the radioactive phosphate was washed out from each bath with a total of five bath volumes of PSS. A further 1 hr incubation was performed with 5×10^{-5} M hydralazine added to the second organ bath. After this final incubation period, both tissues were challenged with 10^{-6} M noradrenaline. After 4 min, the tissues were rapidly removed (within 1 sec) and frozen in liquid N_2. The frozen tissues were dissected into small pieces and homogenized in 30 ml of 3% (w/v) perchloric acid using a Brinkmann polytron homogenizer and the suspension centrifuged at 40,000 *g* for 20 min. The supernatant was used to quantitate the non-protein phosphate pool. The protein residue was washed once with 30 ml, 2% trichloroacetic acid containing 5 mM sodium dihydrogen phosphate, dissolved in 0.25 M sodium phosphate buffer pH 8.0, 2% SDS and dialysed overnight against 10 mM Tris, pH 7.5, 1% (w/v) SDS, 1% (w/v) mercaptoethanol.

Samples containing equi-amounts of protein were analysed by SDS–PAGE as described above, stained for protein with Coomassie Blue RL, destained and dried for autoradiography.

RESULTS

Myofibrils prepared by the method of Sobieszek and Bremel [8] were used to investigate the actomyosin ATPase of arterial smooth muscle from bovine carotid arteries. The properties of this preparation were similar to those of arterial actomyosin [12, 13].

The stimulatory effect of Ca^{2+} on actomyosin ATPase and phosphorylation is shown in Fig. 1. The activation by Ca^{2+} of the phosphorylation of myofibril proteins closely followed that of the actomyosin ATPase. The half maximal stimulation

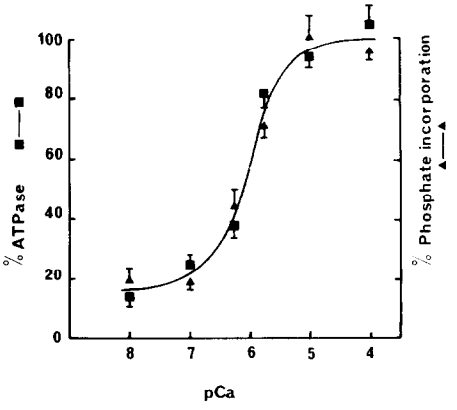

Fig. 1. The dependence of the myofibril ATPase and phosphorylation on Ca^{2+}. The ATPase and phosphorylation was assayed as described under Experimental Procedures. Ca^{2+} concentrations were maintained by using EGTA to buffer free Ca^{2+}. Phosphorylation was assayed as trichloroacetic acid insoluble material. Activity is expressed as a percentage of that at 5×10^{-5} M Ca^{2+}. Myofibril ATPase, ■; myofibril phosphorylation, ▲. Bars indicate S.E.

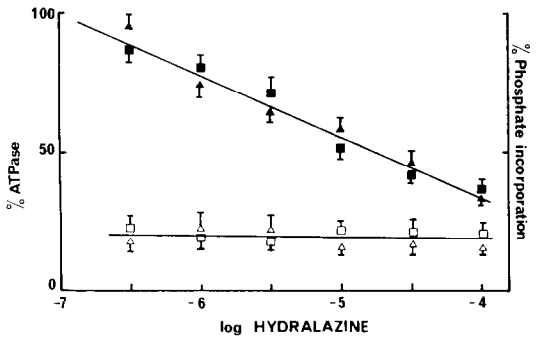

Fig. 2. The effect of hydralazine on carotid arterial myofibril ATPase and phosphorylation. ATPase activity and phosphorylation were measured as described under Experimental Procedures at 10^{-8} M Ca^{2+} (open symbols) and 5×10^{-5} M Ca^{2+} (solid symbols) in the presence of various concentrations of hydralazine. Activity is expressed as a percentage of the ATPase or phosphorylation at 5×10^{-5} M Ca^{2+} without hydralazine. Phosphorylation, ■, □; ATPase activity, ▲, △.

of both activities occurred at the same concentration, namely 10^{-6} M. A maximum fivefold stimulation over the basal activity at 10^{-8} M, was found at 5×10^{-5} M Ca^{2+}. Complete removal of Ca^{2+} with EGTA resulted in a 90% inhibition over the maximum activity (not shown). At 5×10^{-5} M Ca^{2+}, the maximal phosphorylation corresponded to approximately 2 moles phosphate per mol of myosin light chains. These results, therefore demonstrate that the

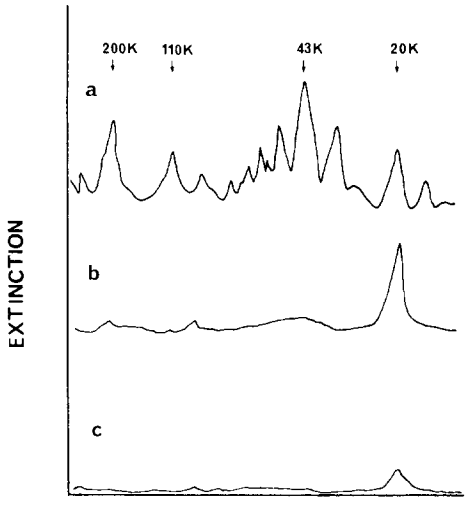

Fig. 3. Comparison of the distribution of {^{32}P} labelled proteins from the untreated and hydralazine treated myofibrils. A myofibril preparation was incubated with 1 mM {^{32}P} ATP with and without hydralazine, the reaction was quenched with perchloric acid and processed for gel electrophoresis and autoradiography as described under Experimental Procedures. Densitometry tracing of (a) coomassie blue stained gel of myofibril protein, densitometry tracing of autoradiograph of (b) myofibril proteins incubated without hydralazine (c) with hydralazine. Arrows show positions of smooth muscle protein makers. Molecular weights obtained from mobilities of myosin P-light chains myosin heaving chains and thin filament proteins: actin and α-actinin.

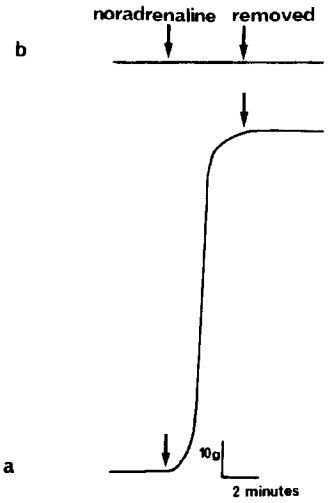

Fig. 4. The effect of hydralazine on the time course of the response of intact arterial strips to noradrenaline, preincubated with {^{32}P} inorganic phosphate. The arterial strips were challenged with 10^{-6} M noradrenaline as shown: (a) untreated; (b) preincubated with 5×10^{-5} M hydralazine for 30 min. Tissues were removed as indicated, frozen and processed as described under Experimental Procedures. The curve shows the expected continuation of the response as established by an identical test dose of noradrenaline.

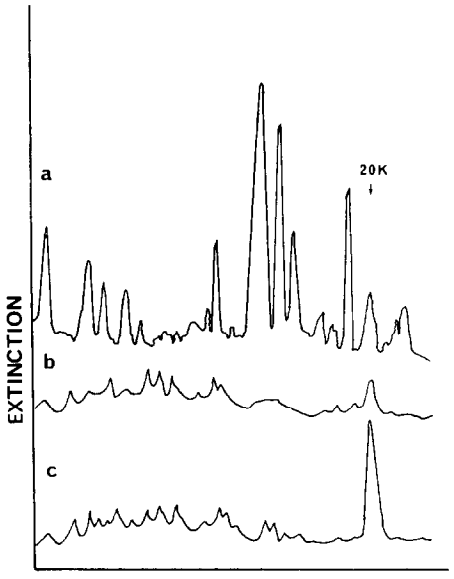

Fig. 5. Comparison of the distribution of {^{32}P} labelled proteins from the untreated and hydralazine treated arterial strips. Densitometry tracing of (a) coomassie blue stained gel of homogenate protein. Densitometry tracing of autoradiograph of {^{32}P} labelled proteins from (b) hydralazine treated strip (c) untreated strip. Muscle strips were preincubated with {^{32}P} inorganic phosphate, washed and then incubated for 30 min in the absence of hydralazine or with hydralazine; both tissues were challenged with 10^{-6} M noradrenaline and after 4 min, removed, frozen and homogenized. Homogenate proteins were analysed by SDS–PAGE and autoradiography.

amount of phosphorylation correlated quantitatively with the Ca^{2+} dependent actomyosin ATPase activity of the myofibrils.

The effect of hydralazine on myofibril ATPase was investigated in incubations with 10^{-8} M and 5×10^{-5} M Ca^{2+}. The experimental results are plotted as the mean of six experiments and both ATPase and phosphorylation fitted to a common regression line ($r = 0.988$, Fig. 2). At low Ca^{2+}, no significant effect on the ATPase or phosphorylation was observed even when the hydralazine concentration was raised to 10^{-3} M. In contrast, at the higher Ca^{2+} concentration of 5×10^{-5} M, hydralazine progressively inhibited both ATPase activity and phosphorylation. Half-maximal effects were found at 1.7×10^{-5} M hydralazine and almost abolished at 10^{-4} M.

The proteins phosphorylated in these same incubations were analysed by SDS–PAGE. The myofibril preparation contained prominent protein bands at molecular weights 20 K, 43 K, 110 K and 200 K (Fig. 3a). These proteins corresponded in mobility to the well established components of smooth muscle, namely myosin P-light chains, actin, α-actinin and myosin heavy chains respectively [8]. The phosphorylation of the myofibril proteins was detected by autoradiography and densitometry (Fig. 3b and c). $\{^{32}P\}$-phosphate incorporation was restricted to the 20 K protein in the incubation of myofibrils with 5×10^{-5} M Ca^{2+} and $\{^{32}P\}$ ATP. Incubation with hydralazine reduced this incorporation by $80 \pm 3\%$ ($N = 6$) (Fig. 3c).

Phosphorylation in intact arterial strips. Two arterial strips were preincubated with $\{^{32}P\}$ phosphate and then one strip was further treated with 5×10^{-5} M hydralazine while the other was left untreated. Both strips were then challenged with noradrenaline and the tension generated was measured. In the case of the hydralazine treated strip, no measurable contraction occurred whereas the other strip contracted with the same initial kinetics as the test dose (Fig. 4). The tissues were rapidly removed 4 min after noradrenaline addition and frozen in liquid N_2.

The phosphate incorporation into smooth muscle proteins was then analysed by SDS–PAGE, autoradiography and densitometry. Figure 5 shows a typical densitometer tracing from an autoradiograph of hydralazine treated and untreated samples.

The predominant phosphate incorporation in the untreated tissue appeared in a protein of 20 K molecular weight, which again, co-migrated with 20 K myosin P-light chains. Preincubation with hydralazine decreased this incorporation by $71 \pm 5\%$ ($N = 6$), whereas incorporation into several other proteins remained unchanged. A similar decrease in phosphate incorporation was found in resting muscle compared with the noradrenaline contracted muscle.

It was not possible to investigate the phosphorylation of myosin P-light chains during hydralazine induced relaxation of noradrenaline contracted strips because of technical problems. Hydralazine requires nearly thirty minutes for the onset of its action by which time the contraction has begun to fade and the phosphorylation to decline.

DISCUSSION

Several studies have concluded that the hypotensive action of hydralazine is mediated by an intracellular action on smooth muscle [1], possibly by a direct effect on actomyosin [17]. This is the first study that directly links hydralazine with an action at the level of the contractile apparatus. Using a myofibril preparation, a well established *in vitro* model of the actin–myosin interaction during contraction, it was demonstrated that the actin-activated myosin ATPase was Ca^{2+} dependent as reported by others [6, 1]. The ATPase activity was also positively correlated with the degree of phosphorylation of a 20 K protein. It is likely, in agreement with previous studies, that this protein is the 20 K myosin P-light chain with which it co-migrates on SDS–PAGE. It therefore follows that the myofibril preparation contained the now, well documented regulatory proteins of smooth muscle contraction, MLCK and calmodulin (see Introduction).

Hydralazine inhibited both the phosphorylation of the 20 K protein and the actin activated Ca^{2+} dependent ATPase, in a concentration dependent way, suggesting a strong correlation between the hydralazine inhibition and the two effects (Fig. 2). The noradrenaline induced contraction of carotid arterial strips was also inhibited by comparable concentrations of hydralazine and with an associated decrease in phosphorylation of the myosin P-light chains. These observations support the hypothesis that hydralazine acts intracellularly on the actomyosin regulatory system to prevent the phosphorylation switch which initiates muscle contraction.

There are already precedents for drugs acting on this regulatory step. The so-called "calmodulin inhibitors" such as trifluoperazine, bind to the Ca^{2+} calmodulin complex to prevent the activation of MLCK [15]. It may be significant that hydralazine also increases cyclic nucleotide levels during its relaxant effect on smooth muscle, since cyclic nucleotide phosphodiesterases are also calmodulin dependent enzymes [16]. Hydralazine may then inhibit myosin P-light chain phosphorylation and increase cyclic nucleotides by a common mechanism, namely by binding to calmodulin. The latter action may also result in vasodilation *in vivo* by a cyclic AMP dependent stimulation of the Ca^{2+}-ATPases involved in Ca^{2+} sequestration and efflux [14].

In view of the findings in this paper, it is unlikely that the hypotensive action of hydralazine is solely due to a decrease in Ca^{2+} influx into smooth muscle [2]. In fact, the range of intracellular Ca^{2+} concentration over which this change was observed, is higher than that found to modulate the actin–myosin interaction. My results suggest that a direct action on the actomyosin system may contribute to the hypotensive action. However, other sites of action *in vivo* cannot yet be dismissed, especially if the drug acts on an ubiquitous molecule like calmodulin. Moreover, the action of hydralazine may not be identical to the "calmodulin inhibitors", particularly as the onset of its effect is so slow. Further investigations using both intact muscle and subcellular components will be required to explain fully its hypotensive actions.

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REFERENCES

1. D. G. Taylor, in *Pharmacology of Antihypertensive Drugs* (Ed. A. Scriabne), p. 407. Raven Press, New York (1980).
2. A. J. McLean, P. du Souich, K. W. Barron and A. H. Briggs, *J. Pharmac. exp. Ther.* **207**, 40 (1978).
3. K. D. Schultz, K. Schultz and G. Schultz, *Nature, Lond.* **265**, 750 (1977).
4. J. Diamond and R. A. Janis, *Nature, Lond.* **271**, 472 (1978).
5. R. A. Murphy, *Ann. Rev. Physiol.* **41**, 737 (1979).
6. A. Sobieszek and J. V. Small, *J. molec. Biol.* **112**, 559 (1977).
7. J. di Salvo, J. E. Gruenstein and P. Silver, *Proc. Soc. exp. Biol. Med.* **158**, 410 (1978).
8. A. Sobieszek and R. D. Bremel, *Eur. J. Biochem.* **55**, 49 (1975).
9. R. J. Solaro and J. S. Shiner, *Circ. Res.* **39**, 8 (1976).
10. Z. Zaman and R. L. Verwilghen, *Analyt. Biochem.* **100**, 64 (1979).
11. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
12. J. Di Salvo, E. Gruenstein and C. Schmidt, *Proc. Soc. exp. Biol. Med.* **162**, 337 (1979).
13. R. Z. Litten, R. J. Solaro and G. D. Ford, *Archs Biochem. Biophys.* **182**, 24 (1977).
14. C. P. Van Breeman, P. Aaronsan and R. Loutzenhiser, *Pharmac. Rev.* **30**, 167–208 (1978).
15. H. Hidaka, T. Yamaki, T. Totsuka and M. Asano, *Molec. Pharmac.* **15**, 49 (1979).
16. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
17. C. L. Seidel, J. C. Allen and R. L. Bowers, *J. Pharmac. exp. Ther.* **213**, 514 (1980).