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An investigation of the actions of diltiazem on rat aorta exposed to acute hypoxia followed by re-oxygenation

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1 The effects of diltiazem and removal of extracellular Ca^{2+} were examined on contractions, of the rat isolated aorta, to noradrenaline (NA) and high K^+, during exposure to oxygenated conditions and hypoxia followed by re-oxygenation.

2 Exposure to hypoxia caused a similar reduction of contractile responses to NA and KCl, while re-oxygenation restored contractile activity.

3 Ca^{2+}-free conditions abolished responses to KCl but a transient response to NA remained which was resistant to hypoxia.

4 Diltiazem produced similar reductions of responses to NA during both oxygenated conditions and hypoxia, whereas during re-oxygenation the effects of diltiazem upon responses to NA were enhanced.

5 Diltiazem produced a more pronounced reduction of responses to KCl than of responses to NA. However, the reduction of responses to KCl by diltiazem was not modified by the changes in Po_2 examined in the present study.

6 The present study indicates that contractions of the rat aorta mediated by intracellular Ca^{2+} are resistant to the hypoxic conditions studied in the present investigation, whereas those responses mediated by an influx of Ca^{2+} are reduced. The increase in the contractile response to NA following re-oxygenation may result from an increased influx of extracellular Ca^{2+} since such responses show an enhanced sensitivity to diltiazem.

Introduction

It has been demonstrated that contractions of vascular muscle are accompanied by an increase in the concentration of free intracellular Ca^{2+} (Ebashi, 1980). The Ca^{2+} responsible for contraction of vascular muscle may arise from mobilization of intracellular stores, or by influx from the extracellular environment through voltage-operated channels activated by membrane depolarization, or through receptor-operated channels activated by agonist-receptor combination (Weiss, 1985).

The contractility of vascular muscle is known to be regulated by changes in local metabolic factors, of which osmolarity, K^+ concentration and oxygen tension are thought to be most important (Sparks, 1980). A reduction in the contractility of vascular muscle during exposure to hypoxia has been demonstrated both *in vivo* (Craigen & Jennett, 1981) and *in vitro* (Detar & Bohr, 1968; Hellstrand *et al.*, 1977; Chang & Detar, 1980). In addition, a rapid recovery of the contractile response on return to normoxia has also been shown (Ebeigbe, 1982). Such alterations in the contractility of vascular muscle during hypoxia and following re-oxygenation have been correlated with a reduction and an increase, respectively, in the uptake of ^{45}Ca (Ebeigbe, 1982).

Since changes in oxygen tension appear to modify the contractility of vascular muscle, the present study was planned to investigate the effects of changes in oxygen tension on the availability of activator Ca^{2+} in vascular muscle. This was achieved by examining the effects of removal of extracellular Ca^{2+} and exposure to diltiazem, a calcium entry blocker.

Methods

Preparations

Cylindrical segements of thoracic aorta (8 mm in length) were obtained from male Wistar rats (200–280 g) killed by cervical dislocation. The preparations were mounted on parallel stainless steel wire holders under a resting tension of 3 g in 20 ml organ baths. Tissues were maintained at 37°C in Krebs solution of

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the following composition (mM): NaCl 118.40, KCl 4.75, $CaCl_2$ 2.50, $MgSO_4$ 1.18, KH_2PO_4 1.19, $NaHCO_3$ 25.00 and glucose 11.66. EDTA (10 μM) and ascorbic acid (50 μM) were also included to reduce the degradation of noradrenaline (NA) (Maxwell *et al.*, 1983).

Po_2 of bathing solutions

Control conditions were achieved by vigorously gassing with 5% CO_2 in O_2 (bath solution Po_2 394 ± 5 mmHg, $n = 27$). Preparations were made hypoxic by gassing with 5% CO_2 in N_2 (bath solution Po_2 76 ± 2 mmHg, $n = 18$). In every series of experiments partial pressures of oxygen were continuously measured from one organ bath using a Clarke type polarographic electrode (Rank Bros., U.K.).

In the present study, gassing of bathing solutions with oxygen or nitrogen mixtures produced lower and higher partial pressures of oxygen respectively than might be expected on theoretical grounds. However, since organ bath covers were not used in this investigation, equilibration of bathing solutions with room air readily accounts for the partial pressures of oxygen observed.

Calcium-free conditions

Preparations were exposed to Ca^{2+}-free conditions by changing to a modified Krebs solution from which Ca^{2+} had been omitted and 0.05 mM EGTA added.

Experimental protocols

Following a 60 min equilibration period, successive responses were obtained to the addition of either 60 mM KCl or 1 μM noradrenaline (NA); these were reproducible to within 10% of each other. These responses could also be reproduced at 45 min intervals over a period of 8 h with less than 10% reduction. Concentration-response curves performed at the beginning of this series of experiments indicated that 60 mM KCl and 1 μM NA both produced approximately 90% maximal responses.

Investigation of tissues under control conditions Responses to NA and KCl were elicited in the presence of diltiazem (10 nM – 10 μM) pre-incubated for 30 min at each concentration. Responses were also elicited in different preparations following exposure to Ca^{2+}-free conditions for 4 min and with Ca^{2+}-free conditions maintained during the response (EGTA-resistant response) (see Figure 1).

Investigation of hypoxic tissues Following equilibration, preparations were exposed to hypoxic conditions for 30 min before stimulation with either NA or KCl. Hypoxic conditions were maintained for approximately 20 min until peak responses were obtained (see Figure 2, 'hypoxic' response). Without removing NA or KCl, tissues were rapidly re-oxygenated and the tension recorded for a further 30 min (see Figure 2, 'recovery' response). The NA or KCl was then removed from the bathing medium (W, Figure 2) and preparations re-equilibrated for 30 min under control conditions. Further hypoxic and recovery responses were then elicited. In this manner, reproducible hypoxic and recovery responses to NA or KCl could be elicited over a period of 8 h with less than 10% reduction. The effects of diltiazem were studied by including this agent in the hypoxic bathing medium for the 30 min before stimulation and whilst recording responses. In separate experiments, calcium was removed from the bathing medium for the final 4 min of hypoxic incubation in order to record EGTA-resistant responses. In such cases, calcium-free solutions were pre-equilibrated at 37°C under hypoxic conditions.

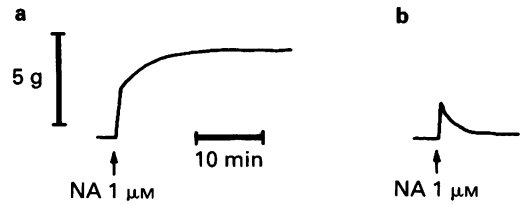

Figure 1 Representative traces of contractions of the rat aorta to noradrenaline (NA) 1 μM in (a) 2.5 mM Ca^{2+} and (b) 0 Ca^{2+} + 0.5 mM EGTA (EGTA-resistant response). Time and tension scales apply to (a) and (b).

Statistical analysis

Values are presented as arithmetic means with the associated s.e.mean for the number (n) of experiments noted. Statistical evaluation of results was carried out by use of Student's unpaired t test. Values of $P < 0.05$ were considered significant.

Drugs and chemicals

Noradrenaline bitartrate and EGTA-sodium salt were obtained from Sigma. Diltiazem hydrochloride was a gift from Synthelabo. All other agents were obtained from BDH.

Results

The effects of changes in Po_2 upon contractile responses

Exposure of the rat aorta to hypoxia caused a similar reduction of control responses to NA and KCl (28.0 ± 2.7%, $n = 168$ and 25.8 ± 3.7%, $n = 84$, res-

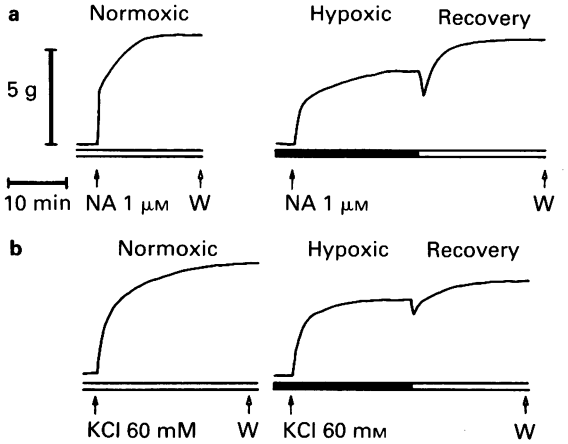

Figure 2 Representative traces of contractions of the rat aorta to (a) noradrenaline (NA) 1 μM and (b) 60 mM KCl during control conditions, hypoxia and following re-oxygenation (recovery). Hypoxic preparations were exposed to hypoxia for 30 min before addition of NA or KCl. Time and tension scales apply to (a) and (b).

pectively). Upon re-oxygenation, a rapid but transient loss of tone was observed, amounting to 33.6 ± 1.6% ($n = 168$) and 16.2 ± 1.1% ($n = 84$) of the control hypoxic response to NA and KCl, respectively (see Figure 2). Continued re-oxygenation produced a recovery of tone reaching 102.7 ± 3.6% ($n = 168$) and 88.4 ± 3.8% ($n = 84$) of the control response to NA and KCl respectively obtained under control conditions. Examples of hypoxic and recovery responses to both NA and KCl are shown in Figure 2. Pilot experiments demonstrated that responses to NA and KCl during hypoxia and re-oxygenation were unaffected by 1 μM propranolol or 10 μM indomethacin ($n = 6$ in each case). These conditions would be expected to reduce the likelihood of β-adrenoceptor stimulation and prostaglandin synthesis respectively.

The effect of Ca^{2+}-free conditions upon responses

Exposure of preparations to Ca^{2+}-free solutions containing 0.5 mM EGTA for 4 min under control condition prevented any response to KCl. However, under such conditions a transient contraction to NA remained (the EGTA-resistant response, see Figure 1), amounting at peak to 41.6 ± 1.7% ($n = 47$) of the peak contraction to NA in the presence of calcium. The EGTA-resistant response to NA was unaffected by exposure to hypoxia for 30 min (0.9 ± 3.2% reduction, $n = 12$). Re-oxygenation at any time during the EGTA-resistant response to NA was without effect.

The effects of diltiazem upon contractile responses

Diltiazem produced a concentration-dependent

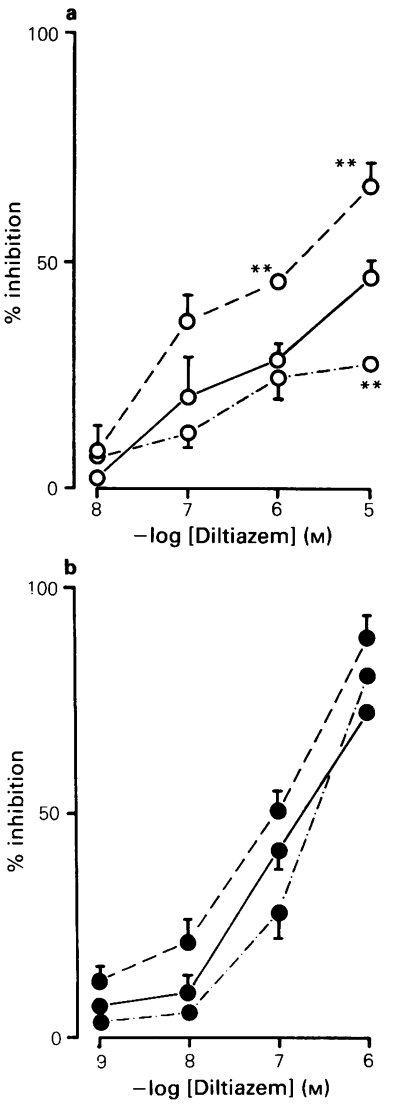

Figure 3 The effects of diltiazem on control (·---·), hypoxic (——) and recovery (– – –) responses of the rat aorta to (a) 1 μM noradrenaline (NA) ($n = 6$, ○) or (b) 60 mM KCl ($n = 5$–6, ●). **Indicates a significant difference ($P < 0.01$) from the hypoxic result. Vertical lines show the s.e.mean.

reduction of the control, hypoxic and recovery responses to NA. The reduction of the response to NA under control conditions was similar in magnitude to the corresponding reduction of the hypoxic response, except at a diltiazem concentration of 10 μM, which produced a significantly ($P < 0.01$) greater reduction of the hypoxic response (Figure 3a). However, diltiazem (> 100 nM) caused a significantly ($P < 0.01$)

greater reduction of the recovery response to NA than of the corresponding hypoxic or control responses. For example, 1 μM diltiazem produced a 24.6 ± 4.7% ($n = 6$), 28.5 ± 3.8% ($n = 6$), and 45.4 ± 2.3% ($n = 6$) reduction of the control, hypoxic and recovery responses to 1 μM NA respectively (Figure 3a).

By contrast, there was no obvious difference between the reduction of control, hypoxic and recovery responses to KCl by diltiazem (1 nM–1 μM). For example, diltiazem (1 μM) reduced the control, hypoxic and recovery responses to 60 mM KCl by 80.2 ± 1.0% ($n = 6$), 72.0 ± 4.7% ($n = 5$) and 88.7 ± 5.0% ($n = 5$) respectively (Figure 3b). At each concentration, diltiazem produced a more pronounced reduction of responses to KCl than of the corresponding responses to NA.

Discussion

In 1982, Ebeigbe found a correlation between the reduction in the response of the rabbit aorta to 1 μM NA during hypoxia, and a concomitant decrease in ^{45}Ca uptake. A corresponding recovery of the response following re-oxygenation was closely related to an increase in ^{45}Ca uptake of the preparations. As the study by Ebeigbe (1982) indicates that the Ca^{2+} handling of vascular muscle changes during hypoxia and following re-oxygenation, the present investigation was intended to examine further this possibility by manipulation of extracellular Ca^{2+} and by use of diltiazem, in order to estimate the availability of Ca^{2+} for contraction.

In the rat aorta, NA and KCl induce a contraction by different mechanisms; KCl appears to facilitate entry of Ca^{2+} into vascular muscle through voltage-operated channels alone, whereas NA may induce influx of Ca^{2+} through receptor- rather than voltage-operated channels (Van Breemen *et al.*, 1979) and release intracellular stores of this ion (Godfraind & Kaba, 1972).

The effects of Ca^{2+} removal

As removal of extracellular Ca^{2+} abolished responses to KCl, yet allowed a residual contraction to NA (the EGTA-resistant response), the present findings confirm the widely held view that the response of the rat aorta to KCl is mediated exclusively by extracellular Ca^{2+}, whereas the response to NA is also supported by Ca^{2+} from an intracellular source. In the present study, the response to NA seen in the presence of EGTA was not maintained. This indicates that the biphasic response to NA seen in the presence of Ca^{2+} consists of an early component which depends upon Ca^{2+} from an intracellular source, followed by a larger maintained component which principally depends upon Ca^{2+} from the extracellular environment.

Since the EGTA-resistant response was unaffected by the reduction in Po_2 employed it is unlikely that the processes responsible for release of intracellular Ca^{2+} in the rat aorta are affected by mild hypoxia. In contrast, the response to KCl and the component of the response to NA which was dependent upon Ca^{2+}-influx, were found to be attenuated by this mild degree of hypoxia. In the study conducted by McGrath and co-workers (Fasehun *et al.*, 1986) no evidence was obtained to suggest that the interaction of NA with adrenoceptors in rat vascular muscle was modified by exposure to hypoxia. Thus, the present findings support the view of Ebeigbe (1982) and Ebeigbe and co-workers (1980) that mild hypoxia appears, either directly or indirectly, to reduce influx of extracellular Ca^{2+}.

The effects of diltiazem

The percentage reduction in the maintained responses to NA and KCl produced by diltiazem were unchanged by exposure to hypoxia. McGrath & Ugwu (1986) demonstrated that the calcium entry blocker nifedipine, was less effective at reversing NA-induced vasoconstriction of the rat tail artery during hypoxia and suggested that nifedipine-sensitive channels played a diminished role in contractile mechanisms under these conditions. Clearly, the effects of calcium entry blockers upon vascular contractility depend upon tissue Po_2. However, further experiments are needed in order to ascertain the mechanisms involved.

Since recovery responses to NA were more sensitive to diltiazem than the corresponding responses during hypoxic or control conditions, it is likely that the rapid reversal of hypoxic conditions resulted in an enhanced influx of extracellular Ca^{2+}. This proposition is consistent with the observations made by Ebeigbe (1982), in that re-oxygenation of hypoxic rabbit aorta produced an increase in the uptake of ^{45}Ca associated with contraction.

By contrast, the percentage reduction of the response to KCl produced by diltiazem was the same during re-oxygenation as during control or hypoxic conditions, suggesting that the mechanisms responsible for NA- and KCl-induced recovery responses are different. Consequently, since this was the case, it is unlikely that a change in binding of diltiazem to its site of action occurred upon re-oxygenation. Thus, it is reasonable to suggest that an increase in Ca^{2+} influx occurred following re-oxygenation of NA-stimulated preparations either via Ca^{2+} channels, which also operate during control and hypoxic conditions, or through recruitment of an additional population of Ca^{2+} channels which are very sensitive to diltiazem.

Mechanisms responsible for the decrease in contractility during hypoxia

Pittman (1981) briefly reviewed several of the mechanisms that might account for a reduction in vascular contractility during hypoxia. These include the release of an endogenous vasodilator, activation of a specialized oxygen receptor, a reduction in energy supply or a reduction in membrane permeability to ions.

It is unlikely that endogenous vasodilators were responsible for the effects of hypoxia observed in the present study since repeated washing of tissues with hypoxic Krebs solution, which would be expected to remove vasodilators from the tissues, did not alter the responses described above (unpublished observations). Further, since the results from the present investigation indicate that responses of the rat aorta which are mediated by intra- and extracellular Ca^{2+} (see above) are differentially affected by exposure to hypoxia, it is difficult to envisage a mechanism by which a 'specialized O_2 receptor' (Pittman, 1981) could modulate contractility, unless it is linked to a specific Ca^{+}-activation process.

In vascular muscle, preformed stores of high energy phosphates are small and it has been suggested that even basal energy requirements would deplete reserves within minutes (Paul, 1980). However, in pilot experiments performed during the present study, reproducible responses of the rat aorta to NA and KCl could be obtained over a period of 5 h during hypoxia without intervening periods of oxygenation (unpublished observations). Such responses would be expected to reduce progressively if formation of energy were limited by the degree of hypoxia induced in the present experiments. Furthermore, it is unlikely that hypoxia produced irreversible damage to the contractile apparatus in the present study, since contractility was readily restored upon re-oxygenation.

In addition to the reasons for hypoxia-induced depression of vascular contractility outlined by Pittman (1981), it is possible that a reduction in Po_2 directly affects the contractile machinery. Recent studies using tracheal (Stephens *et al.*, 1985) and vascular (Morgan, 1987) smooth muscle indicate that the sensitivity to Ca^{2+} of the contractile apparatus is not constant. Since the present study demonstrated a change in sensitivity to diltiazem of NA-induced recovery responses, it is unlikely that the reduction of contractility observed during exposure to mild hypoxia could be fully explained by a modification of the sensitivity to Ca^{2+} of the contractile machinery.

The results from the present study indicate that the diminished contraction of the rat aorta during mild hypoxia results from a reduction in the influx of extracellular Ca^{2+} and upon re-oxygenation an increase in Ca^{2+} influx results in a recovery of contractility. Furthermore, the present experiments demonstrate that the vascular actions of diltiazem depend upon the Po_2 of the surrounding medium. Such findings are important when designing experiments to detect compounds with calcium entry blocking activity and when predicting therapeutic uses of calcium entry blockers. Further experiments are being performed in order to elucidate the physiological importance of these effects.

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