# Effects of calcium, calcium entry blockers and calmodulin inhibitors on atrioventricular conduction disturbances induced by hypoxia

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1 Effects of hypoxia on atrioventricular conduction were investigated in the Langendorff-perfused isolated heart of the rabbit with various extracellular calcium concentrations ($[Ca^{2+}]$) as well as in the presence of verapamil, nifedipine, N-(6-aminohexyl)-5-chloro-l-naphthalenesulphonamide (W-7) and chlorpromazine.

2 The prolongation of the atrio-His (AH) interval by hypoxia for 7 min was greater with increasing $[Ca^{2+}]_o$ ranging from 1.2 to 5.2 mM. At $[Ca^{2+}]_o$ of over 3.2 mM under hypoxic conditions, AH block of the Wenckebach type was observed in some cases.

3 Verapamil ($5 \times 10^{-8}$M) and nifedipine ($5 \times 10^{-8}$M) caused a significant prolongation of AH intervals before hypoxia. However, the intensity of AH prolongation due to hypoxia was significantly attenuated in the presence of the calcium entry blocker, and AH block was not induced even at 3.2 mM $[Ca^{2+}]_o$.

4 W-7 ($5 \times 10^{-6}$M) and chlorpromazine ($10^{-6}$M) did not affect the AH intervals before hypoxia. The hypoxia-induced prolongation of the AH interval or AH block was prevented in the presence of these drugs.

5 W-5, a chlorine-deficient derivative of W-7, showed no protection against hypoxia-induced AV nodal conduction disturbances.

6 These findings suggest that hypoxia-induced AV nodal conduction disturbance is explained, at least in part, by the electrical uncoupling of nodal cells, probably due to the calcium overload. This conduction disturbance is protected by calcium entry blockers or by calmodulin inhibitors, but the mode of protective action is not the same for these different categories of drugs.

## Introduction

The atrioventricular (AV) node is one of the weakest regions for impulse conduction in the heart under various pathological conditions. Experimental evidence has shown that conduction disturbances such as decremental or inhomogeneous conduction, and reentry can easily occur in the AV node region (Watanabe & Dreifus, 1965; 1966; Mendez, 1982). These conduction disturbances have been mainly attributed to electrophysiological characteristics of individual AV nodal cells, especially to a slow up-stroke velocity of the action potential and slow

recovery of excitability (Mendez, 1982). Recently, the inhibition of electrical coupling between cells has also been proposed as an additional mechanism for AV nodal conduction disturbances under ischaemia, hypoxia, or other conditions inhibiting cellular energy metabolism (Ikeda *et al.*, 1980; De Mello, 1982). Under these conditions, intracellular free calcium concentrations are thought to be elevated (Wojtczak, 1979; Nayler *et al.*, 1979).

In the present study, effects of hypoxia on atrioventricular conduction were examined in the isolated Langendorff-perfused heart of the rabbit in the presence of various extracellular calcium concentrations. The influences of calcium entry blockers

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(verapamil and nifedipine) and calmodulin inhibitors ([N - (6 - aminohexyl) - 5 - chloro - naphthalenesulphonamide (W-7), and chlorpromazine)] on the hypoxia-induced atrioventricular conduction disturbance were also investigated.

The results clarified the causal relationship between cellular calcium kinetics and AV nodal conduction. The possible roles of calmodulin in the genesis of AV nodal conduction disturbance are also discussed.

## Methods

Rabbits weighing 1.5 to 2.0 kg were stunned by a blow on the head and exanguinated through the carotid arteries. The heart was quickly removed and a cannula was inserted into the aorta for Langendorff perfusion. The preparation was perfused at a constant pressure (90 cmH$_2$O) with Krebs-Ringer solution having the following composition (mM): NaCl 120.3, KCl 5.0, CaCl$_2$ 1.2, MgSO$_4$ 7H$_2$O 1.3, NaH$_2$PO$_4$ 1.2, NaHCO$_3$ 24.2 and glucose 5.5. The solution was equilibrated with 95% O$_2$ and 5% CO$_2$ to obtain a pH 7.4 and $P$O$_2$ of over 600 mmHg. The temperature of the perfusate was maintained at 34°C. In experiments with high extracellular calcium concentration ([Ca$^{2+}$]$_o$), CaCl$_2$ was added to the perfusate to obtain a [Ca$^{2+}$]$_o$ of 3.2 and 5.2 mM. The hearts were constantly stimulated at a cycle length of 450 ms through a pair of stainless steel electrodes (interpolar distance 1.0 mm), which was placed on the right atrium close to the sinus node region. Pulses for stimulation were 2 ms in duration and twice the diastolic threshold in intensity.

Two sets of bipolar stainless steel electrodes with an interpolar distance of 1.0 mm were inserted through a small incision made in the right atrium so as to record the His bundle electrogram (HBE) and atrial electrogram in the vicinity of the AV node. The signal was amplified at a frequency response from 100 to 500 Hz with a time constant of 0.03 s and displayed on a digital storage oscilloscope (Tektronix 5223) as well as registered on a pen recorder (Watanabe WR 3001).

On the right atrial and His bundle electrograms the onset of atrial (A) and ventricular (V) deflections and the His spike (H) were identified. From these features, AH and HV intervals were obtained. The AH interval represents the AV nodal conduction time from the atrial tissue near the AV node to the bundle of His, and the HV interval represents the conduction time from the bundle of His to the ventricular tissue.

Experimental protocol was as follows. After an equilibration period of 30 min, hypoxia was produced by changing from normoxic perfusate gassed with 95% O$_2$ and 5% CO$_2$ to a hypoxic one gassed with 95% N$_2$ and 5% CO$_2$ (pH 7.4, $P$O$_2$ 30–40 mmHg). Hypoxic perfusion lasted for 7 min and was followed by reinstitution of normoxic perfusion. After 30 min of the normoxic perfusion, drugs at a given concentration were added to the perfusate. Twenty minutes later, a second 7 min period of hypoxia was induced in the continued presence of the drug. This period of hypoxia was followed by 30 min of perfusion with oxygenated solution including the drug. AH and HV intervals were measured every half minute.

Drugs employed in experiments were verapamil (Eisai Pharmaceutical Co., Ltd., Tokyo Japan), nifedipine (Nippon Chemiphar Co., Ltd., Osaka, Japan), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), N-(6-aminohexyl)-1-naphthalenesulphonamide (W-5), and chlorpromazine (Smith Kline & French Laboratories, Philadelphia, P.A.). W-7 and W-5 were kindly supplied by Prof. Hidaka, and Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical analysis was performed using Student's paired $t$ test, and significance was established at $P < 0.05$. More details of each procedure are given under Results.

## Results

### Effects of hypoxia on AV conduction under various [Ca$^{2+}$]$_o$

At 1.2, 3.2 and 5.2 mM [Ca$^{2+}$]$_o$, hypoxia for 7 min caused a progressive prolongation of the AH interval and second degree heart block eventually ensued, whereas the HV interval was not significantly affected by the hypoxia.

Figure 1 shows an experiment at 1.2 mM [Ca$^{2+}$]$_o$. The initial effect of hypoxia on the AH interval was noticed 2.0–2.5 min after changing to the hypoxic perfusate, and the peak effect was observed 1 min after returning to the normoxic condition. The AH interval was then decreased gradually during the subsequent reoxygenation period and completely returned to the control level within 15 to 20 min. The second and third periods of exposure to hypoxia in the same heart which were preceded by 30 min of reoxygenation, caused approximately identical sequential changes in the AH interval. At this calcium concentration atrio-His bundle conduction was not blocked by hypoxia. Data obtained in 8 hearts are shown in Figure 2.

In our experimental system, an appreciable reduction in $P$O$_2$ value at the aortic cannula tip appeared around 1 min after changing from a normoxic perfusate to a hypoxic one, and reached the plateau level, where the $P$O$_2$ value ranged from 30 to 40 mmHg at 2.1 ± 0.1 min (mean ± s.e., $n$ = 5). Therefore, much of the delay in the onset and the offset of the effects of hypoxia can be attributed to the transit time of the solution from the reservoir to the heart.

At 3.2 mM [Ca$^{2+}$]$_o$, the AH interval before hypoxia was shorter than that at 1.2 mM [Ca$^{2+}$]$_o$. However, the

prolongation of the AH interval induced by hypoxia was significantly greater, and in 3 out of 8 hearts AH block of the Wenckebach type occurred immediately after returning to the normoxic perfusion. During the subsequent reoxygenation period, AH intervals decreased gradually toward their control level as occurred with 1.2 mM $[Ca^{2+}]_o$.

At 5.2 mM $[Ca^{2+}]_o$, the prehypoxic value of the AH interval was similar to that at 1.2 mM $[Ca^{2+}]_o$ but hypoxia caused a most remarkable inhibition of AV nodal conduction. Thus, the prolongation of the AH

interval appeared at about 1 min after exposure to hypoxia, and 3 out of 5 hearts showed AH block of the Wenckebach type at the end of hypoxic period. The maximal increase in the AH interval due to hypoxia in the remaining 2 preparations was significantly greater than at 3.2 mM $[Ca^{2+}]_o$.

### Influence of verapamil and nifedipine

Effects of hypoxia were examined in preparations before and after application of verapamil or nifedipine
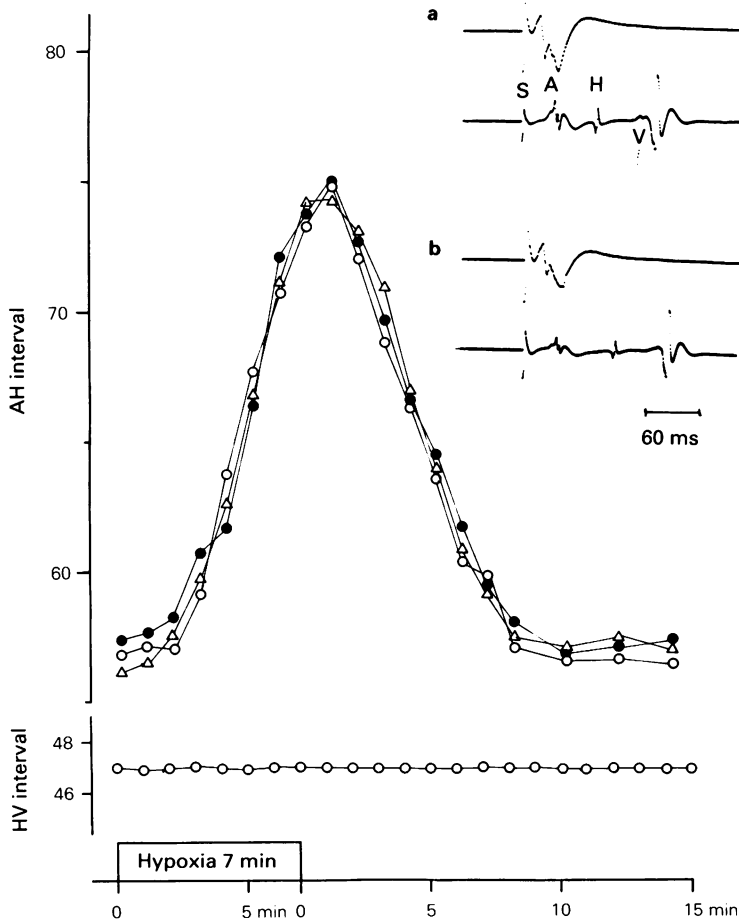


**Figure 1**    Effects of hypoxia on AH and HV intervals in presence of 1.2 mM $[Ca^{2+}]_o$. Upper insets show atrial and His-bundle (HBE) electrograms recorded before (a) and 7 min (b) after the first exposure to hypoxia. The panel is a graphic representation of the experiment. Ordinate scale indicates AH and HV intervals (ms). Abscissa scale shows time (min) from the initiation and termination of hypoxia. In this case, the heart was exposed to hypoxia (7 min) three times, with each exposure followed by a 30 min reoxygenation period. Data obtained during the first (O), second (△) and third (●) exposure were plotted on the same graph. The value of HV interval was stable throughout the experiment, so only the data obtained during and following the first exposure to hypoxia are presented.
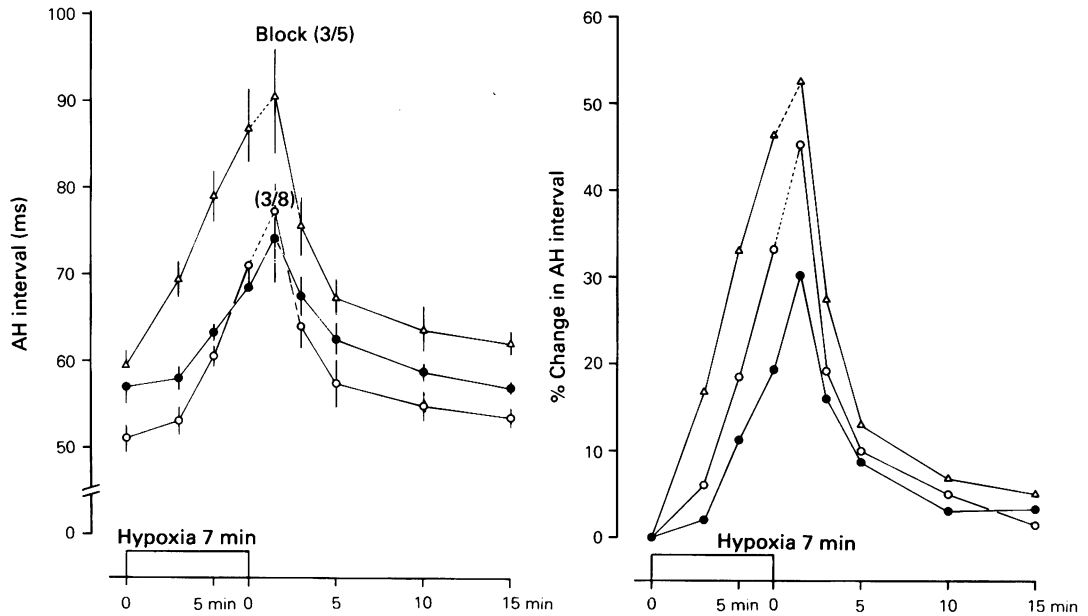
**Figure 2**    Effects of hypoxia on AH interval with various $[Ca^{2+}]_o$. Values obtained at $[Ca^{2+}]_o$ of 1.2 mM (●), 3.2 mM (○) and 5.2 mM (△) are plotted as means (vertical lines show s.e.mean). Ordinates indicate AH intervals (left) and their percentage change from the control immediately before exposure to hypoxia (right). Abscissa scale shows time (min) from the initiation and termination of hypoxia. At 3.2 mM $[Ca^{2+}]_o$ three out of eight preparations showed second degree AH block at the end of hypoxia. At 5.2 mM $[Ca^{2+}]_o$, hypoxia caused a similar AH block in three out of five preparations. In these cases, average values of AH interval were obtained from the remaining preparations.

**Table 1**    Effects of hypoxia on AH interval in the absence and presence of verapamil or nifedipine

| $[Ca^{2+}]_o$ (mM) | | | *Control* (ms) | *Hypoxia* (ms) | *δ AH* (ms) | *AH block* | *Second control* (ms) |
|---|---|---|---|---|---|---|---|
| 1.2 | Untreated | $n = 5$ | 59.0 ± 4.7 | 74.6 ± 10.1 | 15.6 > 6.5 | 0 | 59.8 ± 5.8 |
| | Verapamil | $5 \times 10^{-8}$ M | 63.9 ± 6.7* | 75.2 ± 11.1 | 11.3 ± 4.8* | 0 | 66.1 ± 7.9* |
| | Untreated | $n = 7$ | 53.7 ± 6.1 | 69.8 ± 8.4 | 16.1 ± 4.2 | 0 | 53.1 ± 1.1 |
| | Nifedipine | $5 \times 10^{-8}$ M | 55.9 ± 5.9* | 65.1 ± 6.8* | 9.2 ± 3.8* | 0 | 57.3 ± 5.8* |
| 3.2 | Untreated | $n = 8$ | 48.7 ± 4.0 | 71.4 ± 13.7 | 22.7 ± 9.1 ($n = 5$) | 3/8 | 51.3 ± 4.1 |
| | Verapamil | $5 \times 10^{-8}$ M | 59.3 ± 5.9* | 70.6 ± 7.2 | 11.3 ± 5.4 | 0/8 | 63.2 ± 6.2* |

Data were obtained before (Control) and after exposure to hypoxia for 7 min (Hypoxia) and are presented as means ± s.d. Values for hypoxia are the maximally prolonged AH interval during the early reoxygenation period. δ AH indicates the difference between the values of Control and Hypoxia. The second control was recorded after a 20 min reoxygenation.

*n*: number of preparations. *Significantly different from the values in untreated preparations at respective $[Ca^{2+}]_o$ (*P* < 0.05).

(Table 1). Treatment with verapamil ($5 \times 10^{-8}$M) or nifedipine ($5 \times 10^{-8}$M) for 20 min significantly increased the AH interval under oxygenated conditions at $[Ca^{2+}]_o$ of 1.2 mM or 3.2 mM. Although not shown in the Table, the HV interval was unaffected by these drugs. In the presence of these $Ca^{2+}$ entry blockers, the maximal increase in the AH interval induced by hypoxia was significantly less. At 3.2 mM $[Ca^{2+}]_o$, in three out of 8 untreated preparations hypoxia caused AH block, whereas in the presence of verapamil ($5 \times 10^{-8}$M) hypoxia failed to cause AH block in any preparation.

### Influence of W-7, W-5 and chlorpromazine

Effects of W-7, W-5 (a chlorine-deficient derivative of W-7) and chlorpromazine (Cpz) on the hypoxia-induced AV nodal conduction disturbance were also examined. The results obtained are summarized in Table 2.

Treatment with W-7 ($5 \times 10^{-6}$M), W-5 ($5 \times 10^{-6}$M) or Cpz ($10^{-6}$M) for 20 min did not affect the AH and HV intervals under oxygenated conditions at 1.2 mM $[Ca^{2+}]_o$. At 3.2 mM $[Ca^{2+}]_o$, W-7 ($5 \times 10^{-6}$M) caused a slight prolongation of the AH as well as the HV intervals. The increase in AH interval after exposure to hypoxia was significantly less in the presence of W-7 or Cpz than in their absence. In contrast, W-5 did not affect the prolongation of AH intervals induced by hypoxia. An inhibitory effect of W-7 on the hypoxia-induced AH prolongation was greater at 3.2 mM $[Ca^{2+}]_o$ than at 1.2 mM $[Ca^{2+}]_o$. The AH block, which had been induced by hypoxia in two out of 9

preparations before application of W-7, did not recur in the presence of the drug.

### Discussion

The results show that hypoxia prolonged the AH interval (representing the conduction time from the atrial tissue to the bundle of His) and eventually caused a second degree AH block. In contrast, the HV interval, which represents the conduction time from the bundle of His to the ventricular tissue, was resistant to hypoxia. This high vulnerability of AV nodal conduction to the hypoxic condition is consistent with previous findings (Senges et al., 1979; 1980a,b). Senges et al. (1979) showed that the upstroke phase of the action potential in the AV node was much more sensitive to hypoxia than in atrial muscle. They therefore suggested that hypoxia-induced AV nodal conduction disturbance is mainly attributed to the reduction of $Ca^{2+}$ and/or $Na^+$ inward current through the slow channels, which are known to be affected by cellular energy metabolism. However, they could not eliminate the possible involvement of electrical uncoupling to the conduction disturbance.

Anatomical observations (James & Sherf, 1968; Defellice & Challice, 1969) with electron microscopes have shown that the intercellular connections in the AV nodal region are poorly developed and remarkably few compared to other myocardial tissues. Pollack (1976) demonstrated, using fluorescent dye technique, that intercellular coupling between cells in the central regions of the AV node is much weaker than that

**Table 2**  Effects of hypoxia on AH interval in the absence and presence of W-7, W-5 or chlorpromazine (Cpz)

| $[Ca^{2+}]_o$ (mM) | | | Control (ms) | Hypoxia (ms) | $\delta$ AH (ms) | AH block | Second control (ms) |
|---|---|---|---|---|---|---|---|
| 1.2 | Untreated | $n = 9$ | $54.3 \pm 5.3$ | $70.3 \pm 3.1$ | $16.0 \pm 4.9$ | 0 | $56.7 \pm 5.4$ |
| | W-7 | $5 \times 10^{-6}$M | $57.5 \pm 7.4$ | $67.0 \pm 5.3^*$ | $9.5 \pm 5.0^*$ | 0 | $58.1 \pm 5.1$ |
| | Untreated | $n = 5$ | $56.9 \pm 3.8$ | $75.8 \pm 5.6$ | $18.9 \pm 7.8$ | 0 | $56.0 \pm 2.9$ |
| | W-5 | $5 \times 10^{-6}$M | $59.9 \pm 3.6$ | $78.5 \pm 6.5$ | $18.6 \pm 9.8$ | 0 | $59.9 \pm 4.7^*$ |
| | Untreated | $n = 6$ | $55.1 \pm 1.2$ | $72.7 \pm 4.7$ | $17.6 \pm 9.8$ | 0 | $55.5 \pm 3.8$ |
| | Cpz | $10^{-6}$M | $55.1 \pm 1.5$ | $64.0 \pm 2.6^*$ | $8.9 \pm 4.2^*$ | 0 | $55.9 \pm 3.2$ |
| 3.2 | Untreated | $n = 9$ | $55.3 \pm 5.3$ | $83.4 \pm 14.3$ | $27.9 \pm 14.5$ ($n = 7$) | 2/9 | $56.3 \pm 4.9$ |
| | W-7 | $5 \times 10^{-6}$M | $62.1 \pm 5.7^*$ | $78.6 \pm 9.2^*$ | $16.5 \pm 9.8^*$ | 0/9 | $65.9 \pm 6.9^*$ |

Values are means $\pm$ s.d.
Abbreviations and symbols are the same as in Table 1.

between other myocardial cells. Furthermore, the space constant of the AV node is reported to be considerably smaller than that of atrial fibres (Bonke, 1973), Purkinje fibres (Weidmann, 1952) and ventricular muscle fibres (Weidmann, 1970). This small space constant of the AV node is due to the higher intercellular resistance (De Mello, 1977). These observations suggest that the impulse conduction in the AV node is more susceptible to changes in the intercellular resistance than in other cardiac tissues. In support of this view, Ikeda *et al.* (1980) showed that AV nodal conduction disturbance in the presence of high $[Ca^{2+}]_o$ or ouabain was accompanied by a concomitant increase in the input resistance of AV nodal cells. In ventricular muscle strands, hypoxia was shown to increase the intercellular resistance probably due to the rise in intracellular calcium concentration ($[Ca^{2+}]_i$) (Wojtczak, 1979). Such an elevation of $[Ca^{2+}]_i$ by hypoxia may cause more marked electrical uncoupling in the AV node because of its structural characteristics.

In the present experiments, the hypoxia-induced AH prolongation or AH block was enhanced by an elevation of $[Ca^{2+}]_o$, and prevented by pretreatment with verapamil or nifedipine. These findings also suggest a significant contribution of the electrical uncoupling to the AV nodal conduction disturbance by hypoxia. It has been reported that an elevation of $[Ca^{2+}]_o$ results in an increase in $[Ca^{2+}]_i$ of cardiac cells through an increase in calcium influx (Niedergerke & Orkand, 1966; Reuter, 1967). On the other hand, verapamil and nifedipine at the concentrations used in the present experiments are known to decrease the $Ca^{2+}$ inward current through the slow channels (calcium entry blocker). Accordingly, an elevation of free $[Ca^{2+}]_i$ due to a hypoxia-induced depletion of cellular ATP content is considered to be enhanced with high $[Ca^{2+}]_o$, but restricted in the presence of calcium entry blockers.

Calmodulin, the ubiquitous $Ca^{2+}$-dependent regulator protein (Cheung, 1980), has attracted a great deal of attention in recent years, and is now recognized as the major intracellular protein mediating numerous $Ca^{2+}$-modulated cellular processes, such as cyclic AMP metabolism, glycogen metabolism, cation transport and myosin light chain kinase in the heart (Walsh *et al.*, 1980). The present results showed that hypoxia-induced AH prolongation or AH block was prevented by the pretreatment with W-7 or Cpz, which were reported to be potent calmodulin inhibitors (Levin & Weiss, 1976; Hidaka *et al.*, 1979). In contrast, W-5, which is a less potent calmodulin inhibitor than W-7 (Hidaka *et al.*, 1981), did not show such a protective effect against hypoxia. The concentrations of W-7 and Cpz in the present experiment are similar to their dissociation constants for calmodulin and certainly lie within the range of their effective concentrations for

inhibiting calmodulin-regulated enzyme reactions *in vitro* (Hidaka *et al.*, 1979; Weiss & Wallace, 1980; Means *et al.*, 1982).

There are three possible mechanisms to explain the protective effect of calmodulin inhibitors against the hypoxia-induced AV nodal conduction disturbance. First, analogous to verapamil or nifedipine, calmodulin inhibitors might reduce calcium influx through the slow channels. Recently, Bkaily *et al.* (1984) showed that trifluoperazine (TFP), a potent calmodulin inhibitor, blocks the slow action potential and causes excitation-contraction uncoupling in cultured chick embryonic heart cells. Cpz was reported to inhibit the potassium-induced contraction of vascular smooth muscles by restricting the voltage-dependent calcium influx through the cell membrane (Shibata & Carrier, 1967). In voltage clamp experiments on frog semitendinosis muscle, TFP and W-7 were shown to be effective in blocking the voltage-dependent calcium inward current (Johnson *et al.*, 1982). Based upon these facts, Johnson *et al.* (1982) proposed that calmodulin is a likely candidate as a component or a regulator of the $Ca^{2+}$ channels. However, this proposal has not yet been substantiated and the reduction of calcium influx by these substances through an effect independent of their influence on calmodulin cannot be eliminated. In the present experiments at 3.2 mM $[Ca^{2+}]_o$, the prehypoxic value of the AH interval was significantly increased by W-7. This prolongation might be explained by an inhibitory action of W-7 on the slow calcium inward current of AV nodal cells. At 1.2 mM $[Ca^{2+}]_o$, however, verapamil and nifedipine affected the prehypoxic value of the AH interval but not W-7 or Cpz, indicating no significant inhibition of slow inward current by these substances under these conditions. Accordingly, the protective effects of the calmodulin inhibitors cannot be explained solely by their calcium entry blocking action.

Alternatively, calmodulin may participate directly in the intracellular calcium-mediated process for the electrical uncoupling in the AV node. Peracchia *et al.* (1981) observed that $Ca^{2+}$-induced crystallization of gap junctions isolated from calf lens fibres was inhibited by a specific calmodulin inhibitor, TFP. Furthermore, in experiments on amphibian embryonic cell, calmodulin inhibitors were shown to prevent electrical uncoupling of the cells exposed to $CO_2$ (Peracchia, 1982; Peracchia *et al.*, 1983). These results therefore suggested that cell-to-cell channels of gap junction are occluded by a calmodulin-mediated conformational change in the junctional protein. This assumption might explain the mode of protective action of calmodulin inhibitors on hypoxia-induced AV nodal conduction disturbance.

Finally, calmodulin may play an important role in the deterioration of energy metabolism of cardiac cells

under pathological conditions causing calcium overload. In the experiment of calcium overload-induced heart failure, Schaffer *et al.* (1983) observed that hearts treated with several calmodulin inhibitors including Cpz exhibited less cellular damage than untreated myocardium as reflected by light microscopy, high energy phosphate content, and the loss of protein and creatine phosphokinase into the perfusate. In calcium overloaded cells, mitochondrial damage coupled with the activation of calcium-dependent ATPase, would cause an abrupt decrease in cellular function (Fleckenstein *et al.*, 1974). It has also been suggested that sarcolemmal phospholipase may participate in the damage process by producing excessive lysophosphatides, causing a change in membrane permeability (Franson *et al.*, 1978). Calmodulin is considered to play a role in the activation of these enzymes in the heart (Cheung, 1980). If this is also true for the heart under hypoxic conditions, calmodulin inhibitors could reduce the rate of metabolic and functional deterioration of AV nodal cells causing a conduction failure. Thus, among the three possible explanations presented above, the results obtained from the present experiment seem more reasonably explained by the last two. Further experimental studies, however, are required to define such possible mechanisms.

In conclusion, the present study suggests that hypoxia-induced AV nodal conduction disturbance may be, at least in part, attributed to the electrical uncoupling of nodal cells and that this conduction disturbance is protected by calcium entry blockers or calmodulin inhibitors. Calmodulin inhibitors, unlike calcium entry blockers, do not inhibit the AV nodal conduction under physiological conditions. In this regard, it is expected that calmodulin inhibitors, if they do not have any significant side effects, would be more beneficial in the treatment of heart block due to some metabolic inhibition causing a cellular calcium overload.

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