Effects of pinacidil on the sympatho-adrenal system in dogs

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1 The aim of the present work was to study the antihypertensive effect of pinacidil, a potassium channel opener, in sinoaortic denervated (SAD) conscious dogs and to investigate whether the involvement of the sympathetic nervous system induced by this vasodilator compound is only of baroreceptor reflex origin.

2 Pinacidil (0.1, 0.2, 0.4 mg kg⁻¹ i.v.) induced a dose-dependent decrease in blood pressure in normal as well as in SAD dogs. In contrast, the induced-tachycardia observed in normal dogs was not found in SAD animals.

3 Since pinacidil induced an increase in plasma catecholamines, free fatty acids, glucose, plasma renin activity and aldosterone in SAD dogs, it is suggested that this sympathetic activation is independent of the baroreceptor reflex pathways.

4 The sympathetic activation is mainly of peripheral origin, since pinacidil (0.7 mg kg⁻¹ i.v.) induced an increase in adrenaline release from the adrenal gland after section of the great splanchnic nerve in anaesthetized dogs. This increase is probably due to an effect that does not involve K⁺ channel opening. However, this effect of pinacidil was not observed during splanchnic nerve stimulation (in this case, the involvement of K⁺ channel opening is suggested).

Introduction

Pinacidil, a substituted guanidine, is a new anti-hypertensive agent which produces haemodynamic changes typical of a vasodilator compound acting directly on vascular smooth muscle (Carlsen *et al.*, 1981; Kawashima & Liang, 1985). This drug is one of a new pharmacological class of drugs thought to open apamin-insensitive potassium channels (Weston & Abbot, 1987; Cook, 1988). Kawashima & Liang (1985) suggested that the pinacidil-induced dose-dependent decrease in total peripheral vascular resistance could elicit a secondary sympathetic activation of the baroreceptor reflex and hence explain its positive inotropic and chronotropic effects. The aim of the present work was to study the anti-hypertensive effect of pinacidil in dogs and to investigate whether the pinacidil-induced involvement of the sympathetic nervous system is only of baroreceptor reflex origin. In fact, would pinacidil-induced opening of potassium channels elicit a decrease in sympathetic tone, thus partly explaining its anti-

hypertensive properties? In the present study, two different *in vivo* protocols were used: firstly, chronic neurogenic hypertension in conscious sinoaortic denervated (SAD) dogs, a model deprived of baroreceptor reflex pathways (Damase-Michel *et al.*, 1987), and, secondly, catecholamine release from the adrenal medulla in anaesthetized dogs. For this purpose, the effects of pinacidil on plasma and adrenal catecholamine levels were investigated.

Methods

Protocol 1: Study of pinacidil in conscious SAD dogs

Sinoaortic denervation Eight mongrel dogs of either sex, weighing 11 to 20 kg, were made hypertensive by SAD as previously described (Damase-Michel *et al.*, 1987). Briefly, carotid and aortic nerves were cut under chloralose anaesthesia (80 mg kg⁻¹ i.v.) during two successive procedures, one at time 0 and the

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second one seven weeks later. During the surgery, care was taken to keep intact both vagal and sympathetic fibres in the vagus.

Experimental protocol Three successive doses of pinacidil ($0.1, 0.2, 0.4 \text{ mg kg}^{-1}$) (dissolved in appropriate solvent: NaCl) or vehicle alone were administered at 15 min intervals to two groups of conscious dogs: seven normal (10 to 19 kg body weight) and eight chronic SAD dogs after a resting period of at least 30 min. This protocol was selected according to the data of Kawashima & Liang (1984): cumulative doses were thus $0.1, 0.3, 0.7 \text{ mg kg}^{-1}$ of pinacidil in each dog. For several reasons, it was assumed that the doses used in this study are true cumulative ones. First, the half-life of pinacidil is 2 to 4 h (Word *et al.*, 1984). Moreover, Nicholls *et al.* (1986) and Ward (1984) found that the maximum pinacidil-induced changes were seen a few minutes after injection and lasted for 70 to 180 min. Cardiovascular parameters were measured before and every 5 min for 45 min, plasma catecholamines and free fatty acids at time 0, 15, 30 and 45 min, plasma glucose, aldosterone and renin activity just before (time 0) and at the end of the experiment (time 45 min).

Protocol 2: Study of pinacidil on adrenal medulla in anaesthetized dogs

General procedure Fourteen mongrel dogs of either sex, weighing 15 to 25 kg, were anaesthetized with α -chloralose (80 mg kg^{-1} i.v.), curarized by gallamine (2 mg kg^{-1} i.v.) and artificially ventilated with a Ideal Palmer pump (insufflated air volume: 15 ml kg^{-1} with frequency of 16 min^{-1}). The vagus nerves were cut at the cervical level (C_4). Fully adequate anaesthesia was maintained by an injection of $15\text{--}20 \text{ mg kg}^{-1}$ of chloralose each hour. Body temperature of the animals was maintained at a constant level around 38°C and arterial pH monitored using a Metrohm pH meter.

Arterial venous blood sampling Adrenal venous blood sampling was done according to our previously described technique (Valet *et al.*, 1988a). Briefly, the right adrenal vein was dissected and its collaterals occluded. The animal was heparinized (500 u kg^{-1} every 2 h). The adrenal vein was occluded at the level of its junction with the inferior vena cava, and the other end was cannulated for blood sampling. When not being sampled, adrenal venous blood was returned directly to the femoral vein via a cannula. Blood sampling started 30 min after adrenal vein cannulation. Each blood sample (2 ml) was collected in a tube containing anticoagulant heparin (0.1%) and immediately frozen. Plasma

was immediately separated by centrifugation and stored below -80°C . Adrenal plasma flow rate ($\text{ml kg}^{-1} \text{ min}^{-1}$) was measured and catecholamine output (adrenaline and noradrenaline) expressed as $\text{ng kg}^{-1} \text{ min}^{-1}$.

The right great and lesser splanchnic nerves were dissected, cut below the diaphragm and the surrounding tissue containing sympathetic fibres carefully destroyed in order to obtain a fairly complete denervation. For that reason, we use the term 'adrenal medulla with sectioned great splanchnic nerve' rather than 'denervated'. Section of the splanchnic nerve was made one hour before blood collection was started. The peripheral end of the great splanchnic nerve was stimulated via a bipolar stainless steel electrode. The stimuli consisted of 3 min trains of rectangular pulses of supramaximal intensity (5 V), 0.5 ms duration and frequency 1 Hz. Stimulation was repeated twice for periods of 3 min each separated by 30 min. In the control group, 0.9% NaCl solution (saline) (the vehicle of pinacidil) was given 10 min before the two periods of stimulation and in the pinacidil-treated group, saline or pinacidil was given before the first and the second stimulation periods, respectively. Adrenal venous blood was collected (3 min) before stimulation and 90 s after the beginning of the stimulation.

Drug administration Pinacidil (0.7 mg kg^{-1}) or saline was injected intravenously 10 min before electrical stimulation.

Cardiovascular parameters

Systolic and diastolic blood pressures were recorded by means of a catheter introduced into the abdominal aorta via the left femoral artery and connected to a Gould P23ID transducer on one channel of a Honeywell recorder. Heart rate was obtained by use of a heart period (pulse interval) meter triggered by the electrocardiogram (lead II). Protocol 1 was performed in conscious dogs whereas in protocol 2 the dogs were under chloralose anaesthesia.

Biochemical assays

Plasma or adrenal catecholamines were measured by high performance liquid chromatography using electrochemical detection. Blood samples were obtained from femoral artery for protocol 1 and adrenal vein for protocol 2. Plasma free fatty acids were analysed by the method of Dole & Meinertz (1960). Plasma glucose was determined by an enzymatic method (Biotrol kit, Paris). Plasma renin activity and plasma aldosterone concentrations were measured with commercial radioimmunoassays kits.

Figure 1 Effects of three serial doses of pinacidil (0.1, 0.2, 0.4 mg kg⁻¹ i.v.) on systolic (○), diastolic (●) blood pressures (mmHg), each measured 5 min after drug injection (a, b) and heart rate (beats min⁻¹) measured 15 min after drug injection (c, d) in 7 normal (a, c) and 8 sinoaortic denervated (SAD, b, d) conscious dogs. Statistical analysis was made by Student's *t* test for paired comparisons. Mean values are given; vertical lines show s.e.mean. **P* < 0.05 when compared with pretreatment values. NS = not significant.

Statistical analysis

All data are presented as mean values $\pm$ s.e.mean. Statistical analysis was performed by use of Student's *t* test for paired or unpaired data. The level of significance was *P* < 0.05.

Results

Protocol 1: Study of pinacidil in conscious SAD dogs

Only the results with pinacidil are presented since sham experiments (not shown), using saline as a vehicle, failed to show any significant changes in blood pressure, heart rate, plasma catecholamines and other studied parameters in normal as well as SAD conscious dogs.

Blood pressure and heart rate (Figure 1)

Normal dogs: Pinacidil produced a dose-dependent decrease in systolic and diastolic blood pressures and a simultaneous increase in heart rate. Its peak effects occurred shortly (5 min) after each administration. These acute changes lessened gradually and reached

a steady state within 10 to 15 min after each administration.

SAD dogs: Acute injection of pinacidil (0.1, 0.2, 0.4 mg kg⁻¹) induced a dose-dependent decrease in blood pressure. The time course of this change was similar to that observed in normal dogs. By contrast, pinacidil did not elicit any change in heart rate in conscious SAD dogs during the whole experiment.

Plasma catecholamine levels (Figure 2a and b)

Normal dogs: Under resting conditions (before treatment), plasma catecholamine levels were 289 ± 63 and 187 ± 52 pg ml⁻¹ for noradrenaline and adrenaline respectively. After 0.4 mg kg⁻¹ pinacidil (i.e. cumulative dose of 0.7 mg kg⁻¹) a significant increase was observed.

SAD dogs: Resting values were significantly (*P* < 0.05) higher in SAD than in normal dogs: 418 ± 101 pg ml⁻¹ and 333 ± 107 pg ml⁻¹ for noradrenaline and adrenaline respectively. Pinacidil (0.2 and 0.4 mg kg⁻¹; i.e. cumulative doses of 0.3 and 0.7 mg kg⁻¹) induced a significant increase in plasma noradrenaline whereas 0.1 mg kg⁻¹ pinacidil was

Figure 2 Effects of three serial doses of pinacidil (0.1, 0.2, 0.4 mg kg⁻¹ i.v.) on plasma catecholamines (pg ml⁻¹; a, b) and free fatty acids (mmol l⁻¹; c, d) measured 15 min after drug injection in 7 normal (a, c) and 8 SAD (b, d) conscious dogs. For explanation of analysis of results see Figure 1 legend.

ineffective. The changes in adrenaline levels were only significant after the cumulative dose of 0.7 mg kg⁻¹.

Plasma free fatty acids (Figure 2c and d) As previously found (Valet *et al.*, 1988b), plasma free fatty acid levels were higher in SAD dogs than in normal dogs (821 ± 122 versus 517 ± 57 mmol l⁻¹, $P < 0.05$). In the two groups of animals, pinacidil elicited a significant increase in plasma free fatty acids: these changes were significant after 0.2 and 0.4 mg kg⁻¹ pinacidil (i.e. for cumulative doses of 0.3 and 0.7 mg kg⁻¹).

Plasma glucose, aldosterone and renin activity (Table 1) These values were only measured at time 0 (before the drug) and 45 min after a cumulative dose 0.7 mg kg⁻¹. Pinacidil induced a significant increase in all these parameters in normal as well as in SAD dogs.

Protocol 2: Study of pinacidil on adrenal medulla in anaesthetized dogs

Fourteen mongrel dogs (6 controls and 8 treated animals) were used. Under our experimental conditions (chloralose anaesthesia and gallamine), blood pH (7.42), body temperature (38°C) and haematocrit did not vary. Gallamine was used since it prevented the variations in arterial pressure elicited by respiratory irregularities without inducing ganglioplegy. Sumikawa *et al.* (1979) found that it does not alter the secretion rate of adrenaline from the gland.

Splanchnic nerve stimulation (at a frequency of 1 Hz) significantly increased adrenal catecholamine release in the two groups (controls and pinacidil-treated dogs) (Table 2). Thus, adrenaline secretion was ten to fifteen fold more than the basal value: 10.50 ± 1.66 and 11.87 ± 1.54 ng kg⁻¹ min⁻¹ in controls and experimental groups respectively. A frequency of 1 Hz was chosen since the output (mainly adrenaline) obtained with this frequency was almost identical to the basal value measured when the adrenal nerve was intact: in 18 dogs, the mean basal value of adrenaline secretion was 12.81 ± 1.29 ng kg⁻¹ min⁻¹ (Valet *et al.*, 1988a). Electrical stimulation induced a slight increase in plasma flow rate in the two groups which was only significant in the control group ($9.80 \pm 3.89\%$ of the basal value; $P < 0.05$). This effect could be due to vasoactive intestinal peptide release (Edwards, 1982).

In the control group, the values obtained, before and during the second stimulation, for adrenaline, noradrenaline and adrenal plasma flow rate were not significantly different from the corresponding values observed before and during the first stimulation. Figure 3 shows the ratio $R_{2/1}$ between the values for

Table 1 Effect of pinacidil (cumulative dose: 0.7 mg kg^{-1} i.v.) on plasma glucose, aldosterone and renin activity in normal ($n = 7$) and sinoaortic denervated (SAD) ($n = 8$) conscious dogs

		Normal	SAD
Plasma glucose (mmol l^{-1})	Before	5.2 ± 0.4	4.5 ± 0.3
	After	$6.8 \pm 0.6^*$	$6.4 \pm 0.5^*$
Plasma aldosterone ($\text{ng } 100 \text{ ml}^{-1}$)	Before	14.0 ± 2.8	34.8 ± 12.6
	After	$29.3 \pm 3.7^{**}$	$65.3 \pm 18.6^{**}$
Plasma renin activity ($\text{ng ml}^{-1} \text{ h}^{-1}$)	Before	1.50 ± 0.61	4.44 ± 2.58
	After	$13.49 \pm 3.12^{***}$	$15.51 \pm 3.66^{**}$

Statistical analysis was made by means of Student's *t* test for paired comparisons. Mean values $\pm$ s.e.mean are given.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the second stimulation and the corresponding values for the first period of electrical stimulation. For each parameter, the ratio was around 1, the highest being for noradrenaline (1.17). In this case, the basal values before the second stimulation were not significantly different from the basal values before the first stimulation.

In the treated group, pinacidil was given 10 min before the second period of electrical stimulation. A dose of 0.7 mg kg^{-1} was selected because this dose was found to increase plasma adrenaline levels in conscious SAD. Pinacidil induced a significant increase in the basal secretion (before stimulation) of noradrenaline and adrenaline when compared with control groups (Figure 3). Thus, the ratios $R_{2/1}$ for adrenaline and noradrenaline were 2.16 and 3.96, respectively. In addition, pinacidil significantly raised adrenal plasma flow rate when compared with the control group: the ratio $R_{2/1}$ was 1.51. By contrast during stimulation, pinacidil failed to modify adrenaline and noradrenaline release when compared with the control group (Figure 3). It is important to note

that the ratio $R_{2/1}$ for plasma flow rate (1.48) was similar to the basal value obtained before stimulation.

Discussion

In the present study, the effect of pinacidil, a new potassium channel opener, was studied on sympathetic tone in normal and SAD dogs. This antihypertensive drug is known to have a direct arterial vasodilator effect (Arrigoni Martelli *et al.*, 1980). However, an effect on venous return cannot be excluded as recently described by Grant *et al.* (1989) on the canine saphenous vein using another potassium channel opener (BRL 34915). Moreover, in the model of chronic neurogenic hypertension (SAD), the effectiveness of baroreceptor denervation was checked by the failure of phenylephrine (0.1, 1 and $10 \mu\text{g kg}^{-1}$ i.v.) to induce bradycardia and nitroglycerin (1, 3, 10 and $30 \mu\text{g kg}^{-1}$ i.v.) to induce tachycardia. Thus, the involvement of baroreceptor reflex pathways in the antihypertensive action of drugs in

Table 2 Adrenal catecholamine levels and adrenal venous plasma flow rate before and during the first splanchnic nerve stimulation of the denervated adrenal gland (i.e. before any injection) in control (sham) and pinacidil experiments

	Controls		Pinacidil	
	Before	During	Before	During
Adrenaline ($\text{ng kg}^{-1} \text{ min}^{-1}$)	1.02 ± 0.36	$10.50 \pm 1.66^{***}$	0.82 ± 0.37	$11.87 \pm 1.54^{***}$
Noradrenaline ($\text{ng kg}^{-1} \text{ min}^{-1}$)	0.08 ± 0.03	$1.86 \pm 0.37^{***}$	0.06 ± 0.01	$1.57 \pm 0.30^{***}$
Adrenal plasma flow rate ($\text{ml kg}^{-1} \text{ min}^{-1}$)	0.181 ± 0.019	$0.196 \pm 0.018^*$	0.178 ± 0.01	0.189 ± 0.01

A frequency of stimulation of 1 Hz was applied. Adrenaline and noradrenaline were measured 3 min before and during nerve stimulation. Blood sampling during stimulation was started 90 s after initiation of the stimulus. Each value represents the mean $\pm$ s.e.mean ($n = 6$ for control group, $n = 8$ for pinacidil group). Catecholamine release and adrenal plasma flow rate during stimulation were compared with values obtained before stimulation by means of Student's *t* test for paired comparisons. * $P < 0.05$, *** $P < 0.001$.

Figure 3 Adrenal catecholamine levels (adrenaline: stippled (middle) columns, noradrenaline: solid columns) and adrenal plasma flow rate (open columns) in the control (a) and pinacidil (b) group before and during splanchnic nerve stimulation. Ordinate scale: each value is expressed as the ratio $R_{2/1}$ between the values of the second stimulation and the corresponding values of the first period of electrical stimulation. Mean values in the pinacidil group were compared with the corresponding value from the control group by means of unpaired Student's *t* test. ** $P < 0.01$, *** $P < 0.001$.

conscious dogs can be investigated in this SAD preparation. The main finding in the present study is that, besides the classical involvement of baroreceptor pathways (triggered by the decrease in peripheral resistances, as shown in normal dogs by Kawashima & Liang, 1985), pinacidil induced an unexpected increase in sympathetic tone totally independent of the baroreceptor reflex (as shown by the experiments on SAD dogs). As far as we know, such results have never been described previously.

In fact, the importance of baroreceptor pathways in the chronotropic responses to potassium channel openers has been emphasized. Conflicting results have been obtained: Kawashima & Liang (1985) showed that propranolol only reduced but did not totally suppress the increase in heart rate produced by pinacidil in conscious dogs. In contrast, Cook & Hof (1988) found that the increase in heart rate elicited by BRL 34915 (another vasodilator thought to act by opening potassium channels) was completely abolished by prior administration of bopindolol in normotensive rats. The incomplete abolition of the response by propranolol might be compatible with a chemoreceptor response which has, in dog, a sympathetic component. However, these chemoreceptor reflex pathways are known to be activated when blood pressure dramatically decreases below 80 mmHg or when PO_2 is greatly reduced (for example 15 to 5 kPa), but that was not the case under our experimental conditions.

In order to study the pinacidil-induced variations in sympathetic tone, plasma levels of catecholamines, glucose and free fatty acid were determined. It is well known that catecholamines are powerful lipolytic and hyperglycaemic stimulating agents both *in vivo* and *in vitro* (Berlan, 1976; Milavec-Krisman & Wagner, 1978). These parameters were increased as expected after pinacidil in normal animals (via baroreceptor reflex) but they also rose in SAD dogs (i.e. animals deprived of baroreceptor reflex pathways). The rise in plasma noradrenaline in normal dogs is in agreement with the data of Kawashima & Liang (1985) who found a 66% increase in plasma noradrenaline levels (for a cumulative dose of 0.4 mg kg^{-1}). Finally, an increase in sympathetic tone is also indicated by the rise in plasma renin activity and aldosterone observed in the two groups of dogs.

These results in SAD conscious dogs question the mechanism of the sympathetic activation. The action of pinacidil can be mainly explained by peripheral mechanisms since the drug hardly crosses the blood brain barrier (I. Ahnfelt-Ronne, personal communication). In order to confirm the peripheral origin of this activation, we investigated the effect of pinacidil on splanchnic nerve stimulation-induced catecholamine release, since cells from the adrenal medulla are embryologically analogous to sympathetic autonomic ganglia. First, we want to emphasize that basal values of adrenaline and noradrenaline outputs (around $100 \text{ pg kg}^{-1} \text{ min}^{-1}$) were very low suggesting a fairly complete denervation. We observed that pinacidil induced a significant increase in basal secretion of catecholamines from the gland. This pinacidil-induced increase in adrenal plasma flow rate ($R_{2/1}$ was 1.51) is insufficient to explain the excitatory effect of pinacidil on the function of the adrenal medulla, since the ratios $R_{2/1}$ were 2.16 and 3.96 for adrenaline and noradrenaline respectively. The increase in adrenal plasma flow rate is probably due to the vasodilator properties of pinacidil.

How did the drug induce an increase in plasma and adrenal catecholamines? This effect is unexpected as the opening of K^+ channels, by causing hyperpolarization (Cook, 1988), inhibits calcium influx into the cells where calcium is required for exocytosis of catecholamines; especially sympathetic nerve endings and the adrenal medulla. Consequently, pinacidil should elicit the opposite effect, i.e. a decrease in catecholamine release. Cook (1988) demonstrated that K^+ channel opening reduces beta pancreatic cell electrical activity and inhibits insulin secretion. One possibility is that pinacidil directly affects, in a non-specific manner, the storage sites for catecholamines, as does tyramine (Lindmar *et al.*, 1967). Calcium channel blockers, like nifedipine or

diltiazem, do have such properties (Wolchinsky & Zsoster, 1985; Zsoster & Wolchinsky, 1986). Moreover, this hypothesis of a 'non-specific' effect agrees with a recent communication from Cook *et al.* (1989) who found, in *in vitro* experiments, that the vasodilator activity of pinacidil cannot be solely explained by potassium channel opening. Other less likely mechanisms include the haemodynamic effect of hypotension within the central nervous system, but this is known to be activated only when blood pressure falls below 50 mmHg (Meyer, 1983) and that was not the case under our experimental conditions.

However, the pinacidil-induced release was not observed during splanchnic nerve stimulation. One possible explanation is that this phenomenon was counteracted by an opposite inhibitory effect on catecholamine release. In this case, K⁺ channels may be involved as Wada *et al.* (1987) demonstrated that

adrenal medullary cells have at least three distinct types of K⁺ permeability mechanisms.

In conclusion, we hypothesize that the effects of pinacidil on sympathetic tone are the consequence of three different mechanisms: the first two (baroreceptor reflex and another non-specific direct effect) induce an increase in sympathetic tone, while the third (opening of K⁺ channels) results in a decrease in catecholamine release. The relative importance of these three effects can vary according to physiological circumstances.

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