Relaxation and decrease in [Ca\(^{2+}\)]\textsubscript{i} by hydrochlorothiazide in guinea-pig isolated mesenteric arteries

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1 We examined the effect of the thiazide diuretic, hydrochlorothiazide, on intracellular calcium concentration ([Ca\(^{2+}\)]\textsubscript{i}) and tone in guinea-pig mesentery arteries. Vessels were mounted on a microvascular myograph and loaded with the Ca\(^{2+}\)-sensitive fluorescent dye, Fura-2. 2 Hydrochlorothiazide caused relaxation of noradrenaline-precontracted arteries associated with a fall in [Ca\(^{2+}\)]\textsubscript{i}. Preincubation of arteries with hydrochlorothiazide inhibited both contraction and rise in [Ca\(^{2+}\)]\textsubscript{i} in response to noradrenaline. Hydrochlorothiazide did not affect tone and [Ca\(^{2+}\)]\textsubscript{i} when this was elevated by a combination of depolarizing potassium solution and noradrenaline. 3 Hydrochlorothiazide-induced vasorelaxation and decrease of [Ca\(^{2+}\)]\textsubscript{i}, was abolished by charybdotoxin, a blocker of large conductance Ca\(^{2+}\)-activated K channels. 4 The rise in [Ca\(^{2+}\)]\textsubscript{i}, elicited by caffeine in Ca\(^{2+}\)-free physiological salt solution, and presumably reflecting Ca\(^{2+}\) release from intracellular stores, was not altered by preincubation with hydrochlorothiazide. 5 Under depolarizing conditions hydrochlorothiazide did not alter the relationship between the extracellular concentration of Ca\(^{2+}\) and [Ca\(^{2+}\)]\textsubscript{i}; however, hydrochlorothiazide caused a small reduction in the contraction produced for a given rise in [Ca\(^{2+}\)]\textsubscript{i}, suggesting hydrochlorothiazide may cause a slight desensitization of the contractile machinery. 6 These findings suggest that hydrochlorothiazide opens Ca\(^{2+}\)-activated K channels leading to hyperpolarization and consequent closing of voltage-operated calcium channels. The result of this is an impaired influx of extracellular Ca\(^{2+}\), a decrease in [Ca\(^{2+}\)]\textsubscript{i} and vasorelaxation.

Keywords: Hydrochlorothiazide; diuretics; Ca\(^{2+}\); Fura-2; K channels; calcium channels; vascular smooth muscle

Introduction

Almost four decades after their introduction in 1957, thiazide diuretics are still the mainstream of therapy of a large part of the hypertensive population (Ramsey \textit{et al.}, 1994). Besides the well studied effect of thiazide diuretics on the nephron, little is known concerning direct vascular actions of this class of drug, despite the fact that in the long term, thiazide diuretics act by lowering vascular resistance, rather than by their diuretic effects (Van Brummelen \textit{et al.}, 1980). Hydrochlorothiazide has been reported to relax isolated portal vein by Mironneau and colleagues (1981), and recently relaxant effects of hydrochlorothiazide and related drugs have been demonstrated in human isolated small arteries (Calder \textit{et al.}, 1992; 1993).

It has long been recognized that Ca\(^{2+}\) ions are necessary to trigger and maintain the cascade of protein activation and deactivation that leads to a contractile response in vascular smooth muscle cells. The free concentration of Ca\(^{2+}\) ions that is present in the cytosol of vascular smooth muscle cells governs, to an important extent, the activity of the contractile machinery. The contraction of vascular smooth muscle in response to a vasoconstrictor is initiated by increases in free intracellular calcium ([Ca\(^{2+}\)]\textsubscript{i}). This increase of [Ca\(^{2+}\)]\textsubscript{i} arises from two main sources: influx of extracellular Ca\(^{2+}\) ions, and mobilisation of intracellular calcium stores, probably associated with the sarcoplasmic reticulum (Somlyo & Himpens, 1989). There are also indications that vasoconstrictor agents increase the sensitivity of the contractile machinery to [Ca\(^{2+}\)]\textsubscript{i} (Nishimura \textit{et al.}, 1988; Somlyo & Himpens, 1989; Jensen \textit{et al.}, 1992).

In the opposite case of vasodilatation, [Ca\(^{2+}\)]\textsubscript{i} can decline as a result of blockade of calcium release from intracellular stores or through decrease of influx of extracellular Ca\(^{2+}\), besides the possibility that some relaxants may desensitize the contractile apparatus to Ca\(^{2+}\) (Somlyo & Himpens, 1989).

In view of the key role played by [Ca\(^{2+}\)]\textsubscript{i} in regulating vascular tone, we have studied the interaction between the vasorelaxant, hydrochlorothiazide and [Ca\(^{2+}\)]\textsubscript{i} in isolated small arteries (internal diameter ~300 \textmu m) using the fluorescent indicator, Fura-2. Such arteries were used for this study since they are small enough to contribute significantly to peripheral vascular resistance in vivo (Mulvany & Aalkjaer, 1990) and may better reflect the behaviour of the resistance vasculature than larger upstream vessels.

Methods

Guinea-pig mesentery was removed from male animals (~300 g) killed by cervical dislocation. Resistance vessels (internal diameter 342 ± 15 \textmu m) were dissected free from surrounding tissue and mounted on two 40 \textmu m wires in a single channel myograph dedicated to fluorescence measurements to allow simultaneous measurements of isometric tension (force) and [Ca\(^{2+}\)]\textsubscript{i} to be made.

The myograph contained 5 ml of a modified Krebs-Henseleit physiological saline solution (PSS composition, mM: NaCl 118, KCl 4.7, CaCl\(_2\) 1.5, MgSO\(_4\) 7H\(_2\)O 1.5, NaHCO\(_3\) 25.0, NaH\(_2\)PO\(_4\), 2H\(_2\)O 1.0, Na\(_2\)EDTA 0.03 and glucose 5) maintained at 37°C and aerated with 95% O\(_2\), 5% CO\(_2\). The vessels were allowed to equilibrate for 1 h and then set at a 'normalized' internal circumference 0.9 \(L_{100}\) estimated to be 90% of the circumference they would maintain if relaxed and exposed to 100 mmHg transmural pressure. \(L_{100}\) was calculated for each individual vessel on the basis of the passive length-tension characteristics of the artery and the Laplace relationship (Mulvany & Halpern, 1977). This procedure optimised active force generation by these vessels and
the internal diameters referred to were derived from this calculation.

Prior to the beginning of the studies, vessel viability was assessed by exposing arteries to 118 mM K⁺ solution (KPSS; PSS with equimolar substitution of 118 mM KCl for NaCl), KPSS with noradrenaline (10 μM), and noradrenaline (10 μM) alone. Vessels which did not reproducibly produce tension equivalent to more than 100 mmHg effective pressure (by Laplace) in response to these stimuli were discarded.

A following assessment of vessel viability the vessels were incubated with the fluorescent calcium indicator Fura-2 AM (6 μM) for 2 h in PSS containing 0.02% Pluronic F-127, 0.1% cremophor EL and 0.5% dimethyl sulphoxide to improve loading as previously described (Jensen et al., 1992, 1993). Basal tone and contractile response to noradrenaline and potassium were not affected by exposure to this solution.

Fluorescence was measured with a Deltascan spectrofluorimeter (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an inverted Axiovert 35 fluorescence microscope (Carl Zeiss Oberkochen, Germany). [Ca²⁺], was assessed on the basis of the ratio of fluorescence emission measured at 510 ± 5 nm which was evoked by excitation at 340 and 380 nm. Emission signals and force were measured simultaneously at 4 Hz and acquired on-line using an A/D interface (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an IBM AT PC. Data were stored on an optical disc and later analysed off-line using commercially available software (Photon Technology International Inc., South Brunswick, NJ, U.S.A.). Both changes in force and [Ca²⁺] were normalized by expressing them as a percentage of control responses to KPSS for each individual vessel. Relaxation or fall in [Ca²⁺] was calculated as % reduction in stable active tone or [Ca²⁺], immediately prior to addition of drug.

Guinea-pig mesenteric arteries do not possess tone under resting conditions, therefore the relaxant effect of hydrochlorothiazide was examined on precontracted vessels. In some experiments a supramaximal concentration of noradrenaline (10 μM), or noradrenaline (10 μM) in the presence of KPSS were used to precontract the vessels and a near maximal concentration of hydrochlorothiazide (30 μM) was added once stable tone and [Ca²⁺], were attained. In other experiments we examined the effect of 20 min preincubation with hydrochlorothiazide (30 μM) or vehicle control on noradrenaline-induced rise in tone and [Ca²⁺]. Hydrochlorothiazide had no effect on resting tone or [Ca²⁺]. We also studied the effect of charybdotoxin on hydrochlorothiazide-induced relaxation in vessels precontracted with noradrenaline (10 μM) after pre-incubation with charybdotoxin (100 nM) for approximately 20 min. Charybdotoxin was present throughout these experiments.

The effect of hydrochlorothiazide on release of Ca²⁺ from intracellular stores was studied by examining the effect of caffeine (10 mM) in the presence of extracellular Ca²⁺. Vessels were bathed in Ca²⁺-free PSS (containing 1 mM BAPTA) for 3 min which is sufficient to remove extracellular Ca²⁺, but not to deplete intracellular stores (Garcha & Hughes, 1994). The vessel was then exposed to Ca²⁺-free PSS containing 10 μM caffeine for a further 3 min and then washed out into PSS. Hydrochlorothiazide (30 μM) or vehicle control (0.1% dimethylsulphoxide) was present in PSS and then in Ca²⁺-free PSS for a total of 20 min before addition of caffeine.

The effect of hydrochlorothiazide on the sensitivity of the contractile machinery to [Ca²⁺] was examined by observing the effect of hydrochlorothiazide on contractile and [Ca²⁺] responses to addition of extracellular Ca²⁺ to a nominally Ca²⁺-free high potassium solution. Concentrations of Ca²⁺ referred to as those added to the nominally Ca²⁺-free high potassium solution and extracellular [Ca²⁺] is likely to differ from these. Vessels were bathed in Ca²⁺-free KPSS for 3 min prior to cumulative addition of Ca²⁺ (1 nM–3 mM). Ca²⁺ concentration-response curves (Ca²⁺:1 nM–3 mM) were constructed after either incubation with hydrochlorothiazide for 17 min in PSS prior to changing to Ca²⁺-free KPSS containing hydrochlorothiazide (30 μM) or a similar incubation with vehicle control. In order to exclude time- or order-dependent effects in these studies the order of exposure to hydrochlorothiazide or vehicle was randomized.

**Drugs**

1,2-Bis(2-Aminophenoxy) ethane N',N',N',N' tetraacetic acid (BAPTA), caffeine, cremophor-EL, dimethylsulphoxide, hydrochlorothiazide, noradrenaline bitartrate and verapamil were purchased from Sigma (Poole, Dorset, UK), Pluronic F-127 and pre-weighed aliquots (50 μg) of Fura-2 acetoxymethylester (Fura-2 AM) were purchased from Molecular Probes (Oregon, U.S.A.). One fresh aliquot of Fura-2 AM was used for each experiment. Charybdotoxin was purchased from Peptide Institute (Osaka, Japan). With the exception of charybdotoxin which was stored as frozen aliquots, all drugs were prepared on the day of the experiments. Noradrenaline was dissolved in distilled water and hydrochlorothiazide in dimethylsulphoxide. The final concentration of dimethylsulphoxide 0.1% (v/v) had no effect on vessel reactivity.

**Statistics**

Data are presented as mean ± standard error of mean with the number of observations in parentheses. Data were analyzed by Student’s t test, in the case of paired comparisons, or two-way analysis of variance (ANOVA) in the case of multiple comparisons. P < 0.05 was considered significant. Concentration-response data were fitted to a logistic function:

\[ y = \frac{A + (B - A)}{1 + \left(\frac{x}{10^d}\right)^p} \]

Where \( y \) = effect at a given concentration of drug; \( A = \text{minimum effect} \); \( B = \text{maximum effect} \); \( c = EC_{50} \); \( x = \log [\text{drug}] \); \( D = \text{Hill slope} \), by non-linear regression using Excel 5.0 (Microsoft, U.S.A.) and a macro written by one of us (A.D.H.) on an IBM compatible PC.

**Results**

The effect of hydrochlorothiazide on responses to noradrenaline and the effect of charybdotoxin on hydrochlorothiazide-induced responses

As shown in Figures 1 and 2, the vasorelaxant effect of hydrochlorothiazide (30 μM) was associated with a fall in [Ca²⁺]. In vessels precontracted with noradrenaline (10 μM) in the presence of KPSS, hydrochlorothiazide had no significant effect on vascular tone or [Ca²⁺], (Figures 1 and 2). Inhibition with charybdotoxin (20 min, 100 nM) had no effect on the subsequent contraction to noradrenaline, but under these conditions hydrochlorothiazide-induced relaxation was almost totally inhibited (Figures 1 and 2). This was associated with a marked inhibition of the fall in [Ca²⁺], induced by hydrochlorothiazide; [Ca²⁺], (Figures 1 and 2).

In vessels preincubated with hydrochlorothiazide (30 μM), noradrenaline (10 μM) induced a significantly smaller increase in [Ca²⁺] and force than in vessels preincubated with vehicle control. Force and [Ca²⁺] responses to noradrenaline alone were 69 ± 11% and 50 ± 4% respectively (n = 10); following preincubation with hydrochlorothiazide these were reduced to 29 ± 16% and 35 ± 17% respectively (n = 5; P < 0.05 for force and [Ca²⁺]).
The effect of hydrochlorothiazide on calcium release from intracellular stores

Noradrenaline (10 μM) had no effect on force (−1 ± 2%) or [Ca^{2+}]_i (0 ± 1%) in 3 vessels in Ca^{2+}-free PSS, although endothelin-1 (100 nM) induced a transient rise in force and [Ca^{2+}]_i under the same conditions (n = 2). Preincubation with the calcium antagonist, verapamil (10 μM), also abolished force and [Ca^{2+}]_i responses to noradrenaline (10 μM) in mesenteric arteries (n = 5), though both endothelin-1 (100 nM) and vasopressin (100 nM) induced an increase in tone and a rise in [Ca^{2+}]_i in the same arteries in the presence of verapamil (10 μM). It was therefore not possible to examine the effect of hydrochlorothiazide on noradrenaline-induced release of intracellular stores in this vessel.

Caffeine induced a small and transient increase in [Ca^{2+}]_i, and force of respectively 37.6 ± 12.5% and 20.3 ± 6.3% before and 37.5 ± 21.7% and 18.0 ± 5.6% after hydrochlorothiazide incubation (n = 5 in both cases). The action of caffeine was not significantly affected by the presence of hydrochlorothiazide.

The effect of hydrochlorothiazide on responses to extracellular Ca^{2+} under depolarized conditions: interaction between hydrochlorothiazide and the sensitivity of the contractile machinery

Ca^{2+} concentration-response curves were constructed in the presence and absence of hydrochlorothiazide. Data were analysed in terms of the effect of cumulative addition of extracellular Ca^{2+} on isometric force (Figure 3a), [Ca^{2+}]_i (Figure 3b) and the relationship between isometric force and [Ca^{2+}], derived from the previous two relationships. Hydrochlorothiazide caused a small rightward shift in the extracellular Ca^{2+}-force relationship (Figure 3a); the pD2 for Ca^{2+} in the presence of hydrochlorothiazide was 5.2 ± 0.1 (n = 5), compared with 5.4 ± 0.1 (n = 5) under control conditions (NS), and there was little difference in the relationships between extracellular Ca^{2+} and [Ca^{2+}]_i in the presence or absence of hydrochlorothiazide (Figure 3b); pD2 = 5.4 ± 0.1 (n = 5) in both cases. Examination of the [Ca^{2+}]_i-force relationship indicated a small rightward shift (desensitization) of this curve in the presence of hydrochlorothiazide, although this difference was not found to be statistically significant by 2-way analysis of variance.

Discussion

This study has confirmed that hydrochlorothiazide relaxes guinea-pig isolated mesenteric small arteries as Calier and colleagues (1994) have previously reported. We have shown that this effect of hydrochlorothiazide is associated with a fall in [Ca^{2+}]_i, and it seems likely that this fall in [Ca^{2+}]_i largely...
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Figure 3 The effect of hydrochlorothiazide (HCT; 30 μM) on responses to cumulative addition of Ca²⁺ to nominally Ca²⁺-free depolarized conditions. (a) Concentration-response relationships between added extracellular Ca²⁺ and force in the presence (●) or absence (○) of HCT. HCT was present for a total of 20 min prior to addition of Ca²⁺. Increase in force is expressed as % contraction to KPSS in the same vessel. (b) Concentration-response relationship between added extracellular Ca²⁺ and [Ca²⁺]i in the presence (●) or absence (○) of HCT. Change in [Ca²⁺]i is expressed as % change in [Ca²⁺]i in response to KPSS in the same vessel. (c) Relationship between [Ca²⁺]i and force derived from data in (a) and (b). Each value represents the mean ± s.e.mean of data from 5 separate experiments.

explains the direct vasorelaxant effects of hydrochlorothiazides. Both the reduction in [Ca²⁺]i and force were blocked by charybdotoxin. Charybdotoxin is a 37-amino acid peptide present in the venom of the scorpion Leirus quinquestriatus hebraeus which is a relatively selective inhibitor of large conductance Ca²⁺-activated K⁺ channels (KCa) (Anderson et al., 1988; Gimenez-Gallego et al., 1988). It inhibits K⁺ channels by physically plugging the outer pore of the channel (Miller, 1990), but does not affect sodium or calcium channels (Miller et al., 1985). The finding that charybdotoxin blocks the effect of hydrochlorothiazide on both force and [Ca²⁺]i suggests that these actions of hydrochlorothiazide involve opening of KCa channels to increase K⁺ permeability and hyperpolarize the cell. Such a conclusion is supported by the failure of hydrochlorothiazide to alter tone and [Ca²⁺]i in mesenteric arteries contracted by noradrenaline in the presence of potassium. Under these conditions the vessels will be depolarized and the membrane potential will be near to K⁺ equilibrium potential (probably near 0 mV under these ionic conditions). In this case opening KCa channels would have negligible effect on membrane potential and therefore should not reduce [Ca²⁺], or tone.

Hydrochlorothiazide had no detectable effect on caffeine-induced rises in [Ca²⁺]i or tone. Caffeine is a widely used pharmacological tool which releases Ca²⁺ from intracellular stores (Leijten & Van Breemen, 1984), probably by activating channels in the sarcoplasmic reticulum involved in Ca²⁺-induced Ca²⁺ release (Smith et al., 1988). The failure of hydrochlorothiazide to affect responses to caffeine suggests that hydrochlorothiazide probably does not influence tone or [Ca²⁺]i through an action on the caffeine-sensitive store in these arteries. In contrast, Mironneau (1988) reported that indapamide, a thiazide-like drug, depressed constrictions elicited by release of calcium from the endoplasmic reticulum. However, despite its structural similarities with thiazides there is evidence that indapamide-induced vasorelaxation shows considerable differences from thiazide-induced relaxation and probably acts through unrelated mechanisms (Mironneau et al., 1981; Calder et al., 1992). It was not possible to examine the effect of hydrochlorothiazide on noradrenaline-induced release of intracellular Ca²⁺ in these studies, since noradrenaline had very little or no detectable effect on tone or [Ca²⁺]i in the absence of extracellular Ca²⁺ or the presence of verapamil, a calcium antagonist. Since other agonists, endothelin-1 and vasopressin were able to increase tone and [Ca²⁺]i, under these conditions, these preliminary data suggest that noradrenaline may be unable to mobilize Ca²⁺ from the intracellular store(s) in guinea-pig mesenteric small arteries, though further studies will be necessary to establish this unequivocally.

In our experiments using addition of increasing extracellular Ca²⁺ concentrations to a nominally Ca²⁺-free depolarized solution, we found that hydrochlorothiazide caused a small shift in the relationship between added extracellular Ca²⁺ and force. Although not statistically significant this magnitude of shift is similar to that previously reported by Calder and others (1993), which was interpreted by these authors as indicating a calcium antagonist action of hydrochlorothiazide. Our data where changes in [Ca²⁺]i were measured, do not support such a conclusion and suggest that this modest effect of hydrochlorothiazide is due to a reduction in sensitivity of the contractile apparatus to [Ca²⁺]i rather than interference with Ca²⁺ entry under depolarized conditions. Although such an effect may make some contribution to the vasorelaxant effect of hydrochlorothiazide, overall our data suggest that the effect of hydrochlorothiazide on KCa channels is a more important influence.

A recent study has shown that hydrochlorothiazide increased ⁸⁶Rb⁺ efflux, a marker of K⁺ efflux, from guinea-pig mesenteric arteries (Calder et al., 1994). A similar effect has also been seen using another thiazide drug, cyclothiazide, in tail artery smooth muscle (Moura & Worcel, 1983). Both these findings are consistent with the notion that thiazides increase the permeability of smooth muscle membranes to K⁺, probably by opening KCa. It is not known how hydrochlorothiazide acts on KCa channels. It may be via an intracellular biochemical effect or by a direct interaction with the channel; electrophysiological studies represent the most direct approach to resolving this question.

The possibility that thiazides increase K⁺ permeability may also have implications for thiazide-induced hyperglycaemia. In the pancreas, depolarization of the plasma membrane by inhibition of K⁺ channels and opening of Cl⁻ channels leading to opening of voltage-dependent Ca²⁺ channels and increase of [Ca²⁺]i is involved in the activation of insulin release (Sehlin, 1978; Wollheim & Sharp, 1981; Pedersen & Findlay, 1987). Recently it has been reported that hydrochlorothiazide reduced insulin release by isolated pancreatic β cells and that this was associated with a fall in glucose-stimulated ⁴⁰Ca²⁺ uptake (Sandstrom, 1993). It is known that diazoxide, a drug structurally related to the thiazides, hyper-
polarizes β-cells by opening ATP-dependent K channels (K_{ATP}) and that this impairs insulin release (Malaisse & Malaisse-Lagae, 1968; Henquín & Meissner, 1982; Trube et al., 1986). This effect of diazoxide can be inhibited by agents such as glibenclamide which block K_{ATP} channels and are used as clinically hypoglycemic agents. In contrast, blockers of K_{ATP} channels do not affect thiazide-induced relaxation (Calder et al., 1992), or thiazide-induced 86Rb+ efflux (Calder et al., 1994). Hydrochlorothiazide does not affect the activity of K_{ATP} channels in the β-cell plasma membrane (Gillis et al., 1989), but the effect of thiazide on K_{Ca} channels appears not to have been investigated. In view of our findings and those of others cited above a possible role for K_{Ca} channels is plausible.

In conclusion, we have shown that hydrochlorothiazide-induced vasorelaxation is associated with decreased influx of extracellular Ca^{2+}. This phenomenon is probably mediated through activation of large conductance Ca^{2+}-activated K+ channels, as it was abolished by an antagonist of these channels, charybdotoxin. As a result of these studies we postulate that hydrochlorothiazide opens Ca^{2+}-activated K+ channels, thereby leading to K+ efflux and membrane hyperpolarization, and consequent closing of voltage-operated Ca^{2+} channels and smooth muscle cell relaxation. How hydrochlorothiazide acts on these channels remains to be established.

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