British Journal of Pharmacology (1997) 122, 639–642

Stimulation of intracellular chloride accumulation by noradrenaline and hence potentiation of its depolarization of rat arterial smooth muscle *in vitro*

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1 Double-barrelled ion-selective microelectrodes were used to examine the effects of exogenous noradrenaline upon the membrane potential (E_m) and intracellular chloride concentration ($[Cl]_i$) of arterial smooth muscle from the saphenous branch of the femoral artery of the rat.

2 After treatment with 0.6 mM 6-hydroxydopamine (to functionally denervate the tissue), exogenous noradrenaline (5 nM) caused repeatable depolarization of E_m from -63.7 ± 2.4 mV (s.d., $n=18$) to -53.8 ± 3.4 mV ($P<0.0001$) and increases in $[Cl]_i$ from 31.0 ± 0.5 mM to 42.5 ± 2.2 mM ($P<0.0001$).

3 In the presence of 10 μM bumetanide (an inhibitor of (Na-K-Cl) cotransport), 5 nM noradrenaline caused a depolarization of E_m of 3.0 ± 3.2 mV, and a rise in $[Cl]_i$ of 4.5 ± 2.5 mM.

4 In the presence of bumetanide and 1 mM acetazolamide (used as an inhibitor of a Na-independent inward Cl pump), noradrenaline had no effect on E_m or $[Cl]_i$.

5 In the absence of extracellular chloride, the rise in apparent $[Cl]_i$ in response to 5 nM noradrenaline was abolished but there was a depolarization of 2.0 ± 3.9 mV.

6 These results are consistent with the stimulation of (Na-K-Cl) cotransport and a Na-independent Cl pump by exogenous noradrenaline and with the consequent increase in $[Cl]_i$ and shift in E_{Cl} potentiating the depolarization caused by noradrenaline. The possibility that modulation of $[Cl]_i$ may be a general mechanism of E_m regulation is discussed.

Keywords: Noradrenaline; (Na-K-Cl) cotransport; arterial smooth muscle; intracellular chloride; membrane potential; bumetanide; acetazolamide

Introduction

In rat saphenous arterial smooth muscle, the intracellular chloride concentration ($[Cl]_i$) is substantially higher than would be predicted by a passive Nernstian distribution (Davis, 1992; Davis *et al*., 1993a). This accumulation is the result of the activity of three independent inwardly-directed chloride pumping systems. (Na-K-Cl) cotransport (Davis *et al*., 1993a), Cl:HCO_3 exchange (Davis, 1992) and an acetazolamide-sensitive component, designated here as pump III (Chipperfield *et al*., 1993; Davis, 1996). As chloride is accumulated, the chloride equilibrium potential (E_{Cl}) becomes less negative and therefore chloride has a depolarizing effect on the membrane potential. In chronic rat DOCA/salt hypertension, there are increases in (Na-K-Cl) cotransport and pump III activity. In consequence, $[Cl]_i$ is greater than in normotension and E_m is depolarized (Davis *et al*., 1993a; 1994a).

The changes just described in hypertension are long-term effects and this study was undertaken to investigate whether there is short-term up-regulation of (Na-K-Cl) cotransport and pump III and, if so, whether the consequent increase in $[Cl]_i$ can modulate E_m. Catecholamine neurotransmitters are known to stimulate cotransport activity (Chipperfield, 1986; Haas, 1989) and, therefore, the effect of noradrenaline on the activity of (Na-K-Cl) cotransport was investigated by direct monitoring of $[Cl]_i$ and E_m by electrophysiological methods. Nothing was known about regulation of pump III. Preliminary accounts of this work have been published (Davis *et al*., 1994b; Chipperfield *et al*., 1997a).

Methods

Male Sprague-Dawley rats (120–450 g, Bantin & Kingman, Hull, U.K.) were killed by concussion and cervical dislocation, and sections of the saphenous branch of the femoral artery excised. These were split longitudinally, then functionally denervated by treatment with 0.6 mM 6-hydroxydopamine (6-OHDA) for 10 min at 37°C in an isotonic solution containing 20 μM glutathione (reduced form) and (in mM): NaCl 140, KCl 5, $CaCl_2$ 2 and $MgCl_2$ 2 (Aprigliano & Hermsmeyer, 1976). The tissue was then resuscitated for at least one hour at 37°C in oxygenated physiological saline solution (PSS) containing (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10 and HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) 5 at pH 7.4.

Double-barrelled ion-selective microelectrodes (Aickin, 1981) were used to record from the preparation (for recording configuration and impalement criteria, see Davis, 1992). The microelectrodes were calibrated in PSS containing 153, 15, 1.5 and 0 mM chloride (obtained by mixing normal and chloride-free PSS). Chloride-free PSS contained (mM): Na glucuronate 140, K gluconate 5, Ca gluconate 12.5, $MgSO_4$ 2, glucose 10 and HEPES 5, at pH 7.4). The concentration of calcium was increased to 12.5 mM in order to compensate for binding by glucuronate and gluconate (Aickin & Brading, 1983). Electrodes showing a sensitivity of less than 50 mV/decade change in $[Cl]_o$ before or after impalement were discarded. The values of $[Cl]_i$ given are corrected for the effects of intracellular interference of 4.4 mM (Vaughan-Jones, 1979; Davis *et al*., 1993b) except in Table 3 where the uncorrected data are shown.

Solutions of noradrenaline and 6-OHDA were made shortly before use and kept in the dark to slow the process of oxidation.

Bumetanide was a gift from Leo Laboratories (Buckingham, U.K.). Noradrenaline, glutathione and Na glucuronate were purchased from Sigma (Dorset, U.K.) and K and Ca gluconate from Aldrich (Dorset, U.K.). All other chemicals were purchased from BDH (Dorset, U.K.).

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All values given are means ± s.d. (n = number of observations = number of animals). Significance was assessed by Student's paired or unpaired t test, as appropriate.

Results

All experiments were performed in nominally HCO_3-free PSS in order to abolish any effect of Cl:HCO_3 exchange on $[Cl]_i$ (Davis, 1992) and were preceded by treatment with 6-OHDA in order to produce functional adrenergic denervation of the muscle (Aprigliano & Hermsmeyer, 1976). Early experiments without this pretreatment produced unacceptably variable results, presumably as a result of unpredictable release of endogenous noradrenaline from sympathetic terminals.

Effect of exogenous noradrenaline on E_m and $[Cl]_i$

The concentration of noradrenaline to be used was determined by constructing a concentration-response curve with concentrations from 0.1 to 50 nM (data not shown). At 5 nM the response was large enough to give clear and repeatable results, but without desensitization provided that at least 10 min elapsed between applications of noradrenaline.

Superfusion of the preparation with PSS containing 5 nM noradrenaline caused a depolarization of E_m and an increase in $[Cl]_i$ (Figure 1). In 18 experiments, E_m depolarized from −63.7 mV to −53.8 mV and $[Cl]_i$ increased from 31.0 mM to 42.5 mM with a consequent shift in E_{Cl} (Table 1). Both changes were significant (Table 1).

Effect of bumetanide

The loop diuretic bumetanide, at low concentrations, is a specific inhibitor of (Na-K-Cl) cotransport (Chipperfield, 1986; Haas, 1989). As described previously (Davis *et al*., 1993a), 10 μM bumetanide caused a fall in $[Cl]_i$ and there was a hyperpolarization of E_m (Figure 2). In 18 experiments, $[Cl]_i$ fell from 31.0 mM to 21.8 mM and the mean hyperpolarization was from −63.7 mV to −65.3 mV (Table 1). As before (Davis *et al*., 1993a), both changes were significant (Table 1).

In the presence of 10 μM bumetanide, the effects of 5 nM noradrenaline on E_m and $[Cl]_i$ were attenuated but not abolished (Figure 2). Under these conditions, noradrenaline caused a smaller increase in $[Cl]_i$ from 21.8 mM to 26.3 mM and a smaller depolarization of E_m from −65.3 mV to −62.3 mV (Table 1). Both changes were significant (Table 1).

The change in $[Cl]_i$ was greater than if it were passively distributed according to E_m. Thus, in the case of purely passive Cl distribution, $[Cl]_i$ would be 13.4 mM at an E_m of −65.3 mV and it would increase to 15.0 mM with a depolarization to −62.3 mV.

Effect of bumetanide and acetazolamide

Acetazolamide is best known as a carbonic anhydrase inhibitor but, in the conditions used here, it is selective for pump III (Chipperfield *et al*., 1993; Davis, 1996). When bumetanide and acetazolamide were used together to block cotransport and pump III, $[Cl]_i$ fell to 12.5 mM as described previously (Chipperfield *et al*, 1993). In this condition, Cl is distributed pas-

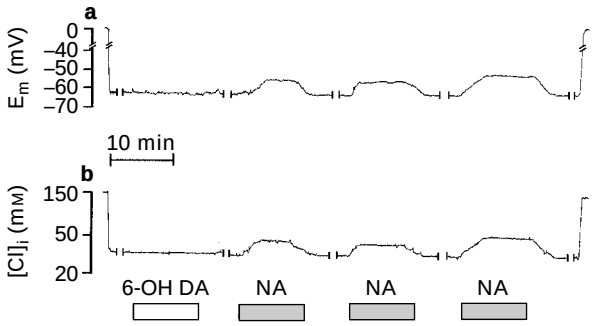

Figure 1 A recording showing the effect of the application of 5 nM noradrenaline (NA) on (a) E_m and (b) $[Cl]_i$ in rat saphenous arterial smooth muscle. The effect was repeatable without desensitization provided that the interval between applications was greater than 10 min (6-OHDA ≡ 6-hydroxydopamine).

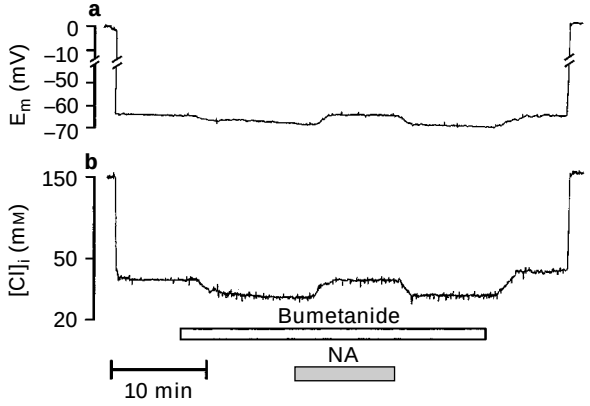

Figure 2 Effect of noradrenaline in the presence of bumetanide on (a) E_m and (b) $[Cl]_i$. Bumetanide caused a hyperpolarization of E_m and a fall in $[Cl]_i$. Noradrenaline (NA) 5 nM depolarized E_m and raised $[Cl]_i$ in the presence of bumetanide but the magnitude of the changes was reduced.

Table 1 Effect of noradrenaline and bumetanide on E_m and $[Cl]_i$ in rat arterial smooth muscle

	E_m (mV)	$[Cl]_i$ (mM)	E_{Cl} (mV)
Control	−63.7 ± 2.4	31.0 ± 0.5	−42.6
+ Noradrenaline, 5 nM	−53.8 ± 3.4	42.5 ± 2.2	−34.2
Difference from control	9.9 ± 4.2	11.5 ± 2.3	8.4
Number	18	18	
Significance	$P < 0.0001$	$P < 0.0001$	
+ Bumetanide, 10 μM	−65.3 ± 2.2	21.8 ± 0.8	−52.0
Difference from control*	1.6 ± 3.3	9.2 ± 0.9	
Number	18	18	
+ Noradrenaline and bumetanide	−62.3 ± 2.3	26.3 ± 2.4	−47.0
Difference from + bumetanide	3.0 ± 3.2	4.5 ± 2.5	5.0
Number	18	18	
Significance	$P < 0.0001$	$P < 0.0001$	

The results are shown as mean ± s.d. and significance was assessed by Student's paired t test and all $[Cl]_i$ are corrected for intracellular interference (see Methods). *Significantly different from control ($P < 0.001$): this has been shown previously (Davis *et al*., 1993a).

sively according to E_m. When noradrenaline was then applied in addition (Figure 3), E_m and $[Cl]_i$ did not change (Table 2).

Effect of noradrenaline in chloride-free medium

If the depolarization induced by noradrenaline depends on the increase in $[Cl]_i$ caused by activation of cotransport, then it should be attenuated in the absence of Cl. On switching the superfusing solution from normal to chloride-free PSS, E_m hyperpolarized and $[Cl]_i$ fell to an apparent level of 4.4 mM, in good agreement with the level determined previously for intracellular interference (4.3 ± 0.6 mM ($n=20$), Davis *et al*., 1993b). The application of 5 nM noradrenaline under these conditions produced a significant depolarization of E_m but it was much smaller than in the presence of Cl (Figure 4). The mean depolarization of E_m was from -69.4 mV to -67.4 mV. The apparent $[Cl]_i$ rose from 4.4 mM to 5.1 mM but this was not significant (Table 3).

Discussion

The results show that exogenous noradrenaline causes an 11.5 mM increase in $[Cl]_i$ and a 9.9 mV depolarization of E_m in rat arterial smooth muscle and that both effects are greatly attenuated in the presence of bumetanide or absence of Cl and abolished in the presence of bumetanide and acetazolamide. The simplest explanation of these observations is that noradrenaline increased the inward pumping of Cl by (Na-K-Cl) cotransport and pump III and that, as $[Cl]_i$ and $[Cl]_o$ have become closer, the noradrenaline-induced shift in E_{Cl} from -43 mV to -34 mV can account for the depolarization. However, this explanation is incomplete in two respects. Firstly, in the absence of Cl, there was a small depolarization of E_m when noradrenaline was applied. Clearly, this cannot be ascribed to Cl and it must be due to some other action of noradrenaline. Secondly, knowing the relative permeabilities of Na, K and Cl (Chipperfield *et al*., 1992a), the noradrenaline induced increase in $[Cl]_i$ from 31 to 42 mM should depolarize E_m by only 1 mV. Therefore, if $[Cl]_i$ is important in modulating E_m, then noradrenaline must have increased the Cl permeability and inward Cl pumping together. In fact, noradrenaline activates Ca-dependent Cl channels in a number of smooth muscles (Large & Wang, 1996) and the increase in inward Cl pumping will not only offset the outward leak through these channels, so as to prevent the dissipation of the Cl gradient, but also potentiate it.

There is clearly evidence for simultaneous activation of cotransport and opening of Cl channels in secretory epithelia. For example, in dogfish and shark rectal glands, NaCl secretion is stimulated by VIP which opens Cl channels at the apical surface and activates cotransport at the basolateral surface

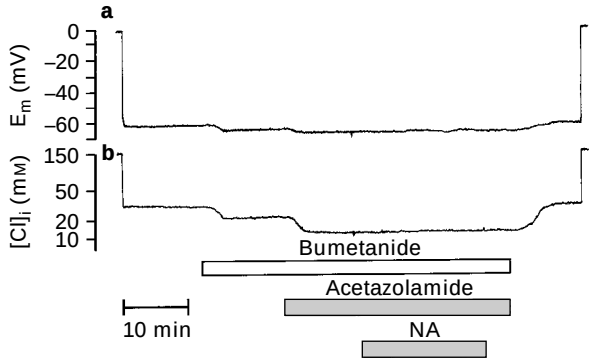

Figure 3 Effect of noradrenaline in the presence of bumetanide and acetazolamide on (a) E_m and (b) $[Cl]_i$. With bumetanide and aceazolamide, $[Cl]_i$ fell to equilibrium with E_m and noradrenaline (NA) had no effect.

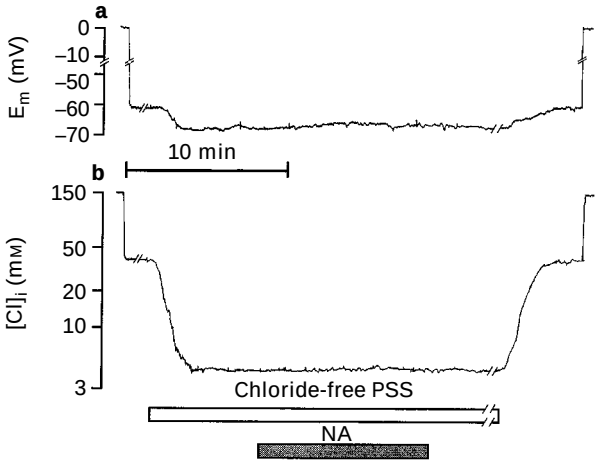

Figure 4 Effect of noradrenaline on (a) E_m and (b) $[Cl]_i$ in chloride-free PSS. Upon removal of extracellular chloride, E_m hyperpolarized, and $[Cl]_i$ fell to a level consistent with intracellular interference (Davis *et al*., 1993b). Noradrenaline (NA) 5 nM caused no change in the apparent $[Cl]_i$, but there was a small depolarization.

Table 3 Effect of noradrenaline on E_m and apparent $[Cl]_i$ in rat arterial smooth muscle in Cl-free media

	E_m (mV)	$[Cl]_i$ (mM)
Control	-63.0 ± 2.9	35.8 ± 1.5
Chloride-free	-69.4 ± 2.5	4.4 ± 0.2
Cl-free + noradrenaline	-67.4 ± 3.0	5.1 ± 2.1
Difference from Cl-free	2.0 ± 3.9	0.7 ± 2.1
Number	14	14
Significance	$P<0.0001$	NS

The results are shown as mean $\pm$ s.d. and significance was assessed by Student's paired t test. $[Cl]_i$ are not corrected for intracellular interference of 4.4 mM (see Methods). NS: not significant.

Table 2 Effect of noradrenaline on E_m and $[Cl]_i$ in the presence of bumetanide and acetazolamide in rat arterial smooth muscle

	E_m (mV)	$[Cl]_i$ (mM)	E_{Cl} (mV)
Control	-63.3 ± 1.3	30.5 ± 0.6	-43.1
+ Bumetanide and 1 mM acetazolamide*	-68.5 ± 1.7	12.5 ± 0.6	-66.9
Ditto + noradrenaline	-67.8 ± 2.4	12.5 ± 0.6	-66.9
Difference due to noradrenaline	0.7 ± 2.9	0.0 ± 0.0	0
Number	4	4	
Significance	NS	NS	

The results are shown as mean $\pm$ s.d. and significance was assessed by Student's paired t test and all $[Cl]_i$ are corrected for intracellular interference (see Methods). *Significantly different from control ($P<0.001$): this has been shown previously (Chipperfield *et al*., 1993). NS: not significant.

(Greger *et al*., 1988; Lytle & Forbush, 1992). Moreover, in dog trachea the same two effects are elicited by adrenoceptor agents (Haas *et al*., 1993). However, whilst the role of co-transport in setting E_m is recognised and the regulation of cotransport is well known (Chipperfield, 1986; Haas, 1989), E_m is not usually considered in work of this kind. Consequently, the modulation of E_m via modulation of $[Cl]_i$ *via* modulation of (Na-K-Cl) cotransport combined with the opening of Cl channels may appear to be new. In fact, the experiments were designed to illustrate what must be true: namely, that regulation of (Na-K-Cl) must affect $[Cl]_i$ and hence E_m also. On the other hand, the observation that short-term up-regulation of pump III can also contribute to the modulation of E_m by a similar mechanism is a new finding.

The question is: do the present observations point to a more general mechanism by which E_m may be adjusted via $[Cl]_i$? Certainly, with regard to (Na-K-Cl) cotransport, a reasonable case can be made. This is because it pumps Cl into many excitable cells, for example, cardiac cells (Liu *et al*., 1989), squid axon (Russell, 1983) and dorsal root and sympathetic ganglia (Ballanyi & Grafe, 1985; Alvarez-Leefmans *et al*., 1988). Furthermore, many humoral factors regulate both cotransport (Chipperfield, 1986; Haas, 1989) and Cl channels (Greger *et al*., 1988; Haas *et al*., 1993; Large & Wang, 1996). Thus, the possibility that chloride ions may play a general, regulatory role in the determination of E_m and, hence, excitability, via cotransport and Cl channel modulation is clearly plausible. With regard to pump III, its involvement cannot be ascertained because there is not enough information about it at the moment. So far, it has been found in only three other tissues, namely rat ventricle (Chipperfield *et al*., 1997b) and human umbilical and placental arteries (Davis, unpublished observations) and in none of them has the question of regulation been addressed.

If $[Cl]_i$ has a regulatory role in setting E_m, then the question is: is it important? In smooth muscle, it is considered that the Ca-activated Cl current 'may have a minor role in the contractile mechanisms in physiological conditions' (Large & Wang, 1996) and the same may apply to Cl currents in excitable cells. Nevertheless, modulation of $[Cl]_i$ must influence E_m and there are clear parallels between the contractility of the rat arterial smooth muscle employed in this study, on the one hand, and the activity of inward Cl pumps, $[Cl]_i$ and E_m, on the other (Chipperfield *et al*., 1992b; Dubb *et al*., 1994).

We thank the British Heart Foundation and the University of Dundee for financial support and Mrs Lorna Steele for technical assistance.

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(Received May 12, 1997
Revised July 8, 1997
Accepted July 10, 1997)