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Inhibition by sodium nitroprusside of a calcium store depletion-activated non-selective cation current in smooth muscle cells of the mouse anococcygeus

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1 The effects of sodium nitroprusside (SNP) on the non-selective cation current activated in response to intracellular calcium store depletion were studied using the whole-cell patch-clamp technique in single smooth muscle cells isolated from the mouse anococcygeus. Voltage-dependent calcium currents were blocked with extracellular nifedipine, and caesium and tetraethylammonium chloride were used to block voltage-dependent potassium currents. Calcium stores were depleted with caffeine (10 mM), carbachol (50 μM) or cyclopiazonic acid (CPA 10 μM; an inhibitor of the sarcoplasmic reticulum [SR] calcium-ATPase).

2 At a holding potential of -40 mV, both CPA and caffeine activated inward currents which consisted of two clearly distinguishable components; an initial transient current followed by a smaller sustained current. In the case of CPA, the amplitudes of the transient and sustained components were 19.7 ± 2.1 pA and 3.5 ± 0.3 pA respectively, whilst the equivalent values for caffeine were 188 ± 21 and 4.8 ± 0.3 pA. As described previously, the transient current results from activation of a calcium-dependent chloride conductance whilst the sustained current is a non-selective cation current, activated following intracellular calcium store depletion.

3 The muscarinic receptor agonist, carbachol, also activated a transient followed by a sustained current with amplitudes of 238 ± 55 and 4.7 ± 0.5 pA respectively. Superimposed on the sustained current were regular, oscillations of calcium-activated chloride current.

4 Both the transient and the sustained currents activated by CPA were absent in cells pretreated with SNP (10 μM). Application of SNP to a cell following activation of the sustained current by CPA inhibited the current by $88.6 \pm 3.8\%$. SNP (10 μM) did not inhibit the transient current activated by caffeine but abolished the sustained current.

5 SNP (10 μM) had no effect on the initial transient current activated by carbachol (50 μM). However, it did inhibit the oscillations in the inward current. In recordings from cells bathed in extracellular solution containing the chloride channel blocker, anthracene-9-carboxylic acid (A-9-C; 1 mM), carbachol activated only a sustained current. This current was inhibited by $88.1 \pm 6.5\%$ by a concomitant application of SNP (10 μM) and was absent in cells pretreated with the nitrovasodilator.

6 The effects of SNP on the currents activated by caffeine (10 mM) were mimicked by 8-bromo-cyclic GMP (200 μM); thus the nucleotide had no effect on the transient current activated by caffeine but abolished the sustained current. The effects of SNP, but not those of 8-bromo-cyclic GMP, were inhibited by the nitric oxide-sensitive guanylyl cyclase inhibitor, 1H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxaline-1-one (ODQ; 1 μM). ODQ alone produced a significant increase in the size of the sustained current activated by caffeine (7.8 ± 0.7 pA).

7 These findings suggest that SNP activates guanylyl cyclase to inhibit the non-selective cation current activated as a result of intracellular calcium store depletion in mouse anococcygeus cells. Since the non-selective cation current appears to underlie the calcium entry process responsible for maintaining the sustained contractions to agonists in this tissue, this action of SNP may represent an important mechanism by which nitrates relax non-vascular smooth muscle.

Keywords: Anococcygeus (mouse); sodium nitroprusside; cyclopiazonic acid; caffeine; carbachol; ODQ; 8-Br-cyclic GMP; store-regulated calcium entry; non-selective cation current

Introduction

Over the past 6 years it has become apparent that the inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter in a number of non-vascular smooth muscles is nitric oxide (NO) or a closely related compound (Rand & Li, 1995). In such tissues, stimulation of the NANC nerves, or application of an exogenous nitrate such as sodium nitroprusside (SNP) produces potent relaxations, those to nerve stimulation but not SNP being blocked by nitric oxide synthase inhibitors. Whilst it is generally accepted that these relaxations are brought about as a result of the nitrates activating soluble guanylyl cyclase, and as a consequence, increasing the intracellular concentration of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), how this leads to relaxation is less clear. Experiments in preparations of vascular smooth muscle have suggested several possible mechanisms including inhibition of the PLC/IP_3 transduction pathway, enhanced calcium sequestration and/or removal from the cell, direct effects on the contractile proteins, membrane hyperpolarization (Lincoln, 1989; Lincoln & Cornwell, 1993) and inhibition of voltage-operated calcium channels (Clapp & Gurney, 1991). However in non-vascular smooth muscle there have been relatively few such studies, presumably because the controlling influence of endogenous nitrates in non-vascular organs has only recently been realised.

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Anococcygeus muscles of mice and rats have proved useful tissues in which to study the cellular mechanisms involved in nitrergic neurotransmission in non-vascular tissues. Experiments aimed at determining the cellular mechanisms by which nitrates relax these smooth muscles have shown that the drugs have no effect on IP_3 production (Gibson *et al.*, 1994a), do not inhibit voltage-dependent calcium currents (England & McFadzean, 1993) and do not alter membrane potential (I. McFadzean, unpublished observations). However there is evidence that they do reduce the free intracellular calcium concentration (Ramagopal & Leighton, 1989) possibly as a result of increased sequestration of calcium into intracellular stores (Gibson *et al.*, 1994b). At higher concentrations, the nitrates also appear to inhibit directly the contractile proteins (Gibson *et al.*, 1994b).

The lack of effect on voltage-dependent calcium channels mentioned above is perhaps not surprising when one considers excitation-contraction coupling mechanisms in the anococcygeus. Whilst contractions to agonists such as phenylephrine and carbachol are effectively abolished in zero extracellular calcium, they are only slightly inhibited by nifedipine at concentrations which abolish contractions to raised potassium (Gibson *et al.*, 1994b). This suggests that prolonged contractions to agonists rely on calcium entry, but via a route other than voltage-operated calcium channels. Agents such as cyclopiazonic acid (CPA), which deplete intracellular calcium stores by receptor independent mechanisms also produce contractions, mediated primarily as a result of calcium entry (Gibson *et al.*, 1994b), and this has led to the suggestion that in this tissue, calcium entry is coupled to intracellular calcium store depletion, an idea first formalised by Putney (1986) in his capacitative model for receptor-regulated calcium entry. Recently we have described the properties of a non-selective cation current, the pharmacology of which suggests that it may be the pathway for calcium entry initiated during sustained contractions of the anococcygeus to agents which deplete intracellular calcium stores (Wayman *et al.*, 1996a). Since nitrovasodilators are able to relax the sustained tone produced by such agents, this current represents a potential target for the relaxant effects of these drugs. The object of the present series of experiments was therefore to examine the effects of SNP on this non-selective cation current. We used three compounds which deplete the stores of calcium by distinct mechanisms: carbachol, which activates the PLC/IP_3 transduction pathway; CPA, an inhibitor of sarcoplasmic reticulum (SR) calcium ATP-ase; and caffeine, which opens ryanodine-receptor-operated calcium channels on the SR membrane.

Preliminary accounts of some of these results have been published (Wayman *et al.*, 1995, 1996b).

Methods

The methodology for the enzymatic isolation of single smooth muscle cells from the mouse anococcygeus has been described elsewhere (Wayman *et al.*, 1996a). Membrane currents were recorded by the whole-cell variant of the patch-clamp technique. The extracellular solution bathing the cells contained (mM): NaCl 90, tetraethylammonium chloride (TEA) 30, KCl 6, $MgCl_2$ 1.2, glucose 11, HEPES 10, $CaCl_2$ 10, pH 7.20 (with NaOH). When carbachol was used as an agonist, TEA, a weak muscarinic receptor antagonist, was omitted from the extracellular solution. Dihydropyridine-sensitive (L-type) voltage-dependent calcium currents were blocked by the addition of nifedipine (1 μM) to the extracellular solution immediately prior to starting an experiment. The patch-pipette filling solution contained (mM): CsCl 130, TEA 20, HEPES 10, ATP 0.5, GTP 0.5, pH 7.20 (with CsOH). When filled with this solution, patch-pipettes had d.c. resistances of around 6 MΩ.

Currents were recorded and filtered at 1 kHz (−3 dB) using an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, USA), digitised (>3 kHz) using a Digidata 1200 interface (Axon Instruments Inc.) in combination with a personal computer (Mesh 486DX) running pClamp acquisition and analysis software (Axon Instruments Inc.) and stored directly onto the hard disk of the computer. Two continuous records of membrane current were obtained, one filtered at 200 Hz and played onto a thermal chart recorder (Gould TA550) and the other filtered at 1 kHz and recorded with a digital tape recorder (DTR-1200; Biologic, France). All electrophysiological experiments were performed at room temeperature (20–24°C).

Drugs were applied extracellularly by a gravity-fed system incorporating a fine bore (0.28 mm internal diameter) catheter tube placed within 100 μm of the cell under test. Cells were perfused continuously with either drug-free of drug-containing solutions, and switching between drug solutions was achieved by means of solenoid valves. The delay between changing reservoirs and ejection of the drug was approximately 5 s.

The following drugs were used: anthracene-9-carboxylic acid (A-9-C; Sigma); 1,2-*bis* (2-aminophenoxy) ethane *N*, *N*, *N*′, *N*′-tetraacetic acid (BAPTA; Sigma); 8-bromoguanosine 3′, 5′-cyclic monophosphate (8-Br-cyclic GMP; Sigma); caffeine (Sigma); carbachol (Aldrich); cyclopiazonic acid (CPA; Sigma); ethylene glycol-*bis*(β-aminoethyl ether) *N*, *N*, *N*′, *N*′-tetraacetic acid (EGTA; Sigma); nifedipine (Sigma); 1H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxaline-1-one (ODQ; Tocris Cookson)

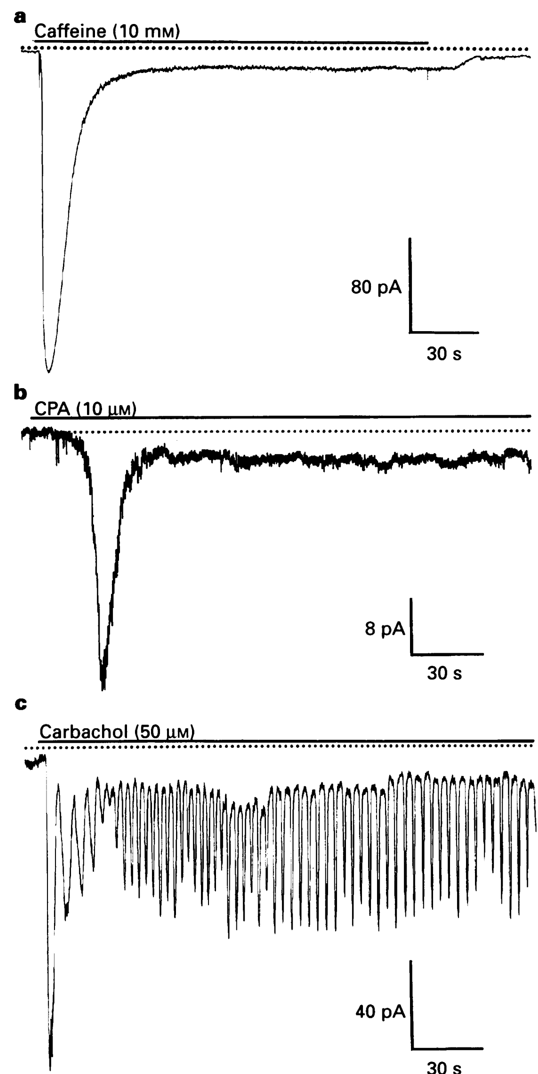

Figure 1 Whole-cell current responses evoked by caffeine, cyclopiazonic acid (CPA) and carbachol in mouse anococcygeus cells. Cells were held at a membrane potential of −40 mV, and zero current is indicated by the dotted line. Drugs were applied for the durations indicated by the solid bars.

and sodium nitroprusside (SNP, Sigma). All salts used were of reagent grade or better. Drugs were prepared as stock solutions in de-ionised water with the exception of CPA and ODQ (10 mM stock in DMSO), nifedipine (10 mM stock in ethanol) and A-9-C (100 mM stock in ethanol).

Results are expressed as mean ± s.e.mean. Statistical analysis was carried out with Student's t test (paired or unpaired where appropriate); $P<0.05$ was taken as significant.

Results

Effect of caffeine, CPA and carbachol on membrane currents

As we have described previously (Wayman *et al.*, 1996a), both caffeine (10 mM) and CPA (10 μM) produced biphasic inward currents when applied to anococcygeus cells held at a membrane potential of −40 mV; an initial, large transient current followed by a smaller, sustained current (Figure 1a, b, Table 1). The transient current is caused by the release of intracellular calcium activating a calcium-dependent chloride current whilst the second current is a non-selective cation current which is activated as a result of depletion of the intracellular calcium stores (Wayman *et al.*, 1996a).

The muscarinic receptor agonist, carbachol (50 μM), produced a qualitatively similar response to caffeine and CPA, with one major exception; superimposed on the second sustained current were regular spikes of inward current (Figure 1c). The oscillations typically had an amplitude of around 80 pA, and occurred at a frequency of 0.27 ± 0.02 Hz ($n=11$). They had a reversal potential of around 0 mV and were abolished by the inclusion of A-9-C (1 mM) in the bathing medium or EGTA (1 mM) in the micropipette-filling solution (data not shown), suggesting that they were produced as a result of activation of the calcium-dependent chloride current.

Table 1 Summary of the electrophysiological responses to caffeine, cyclopiazonic acid (CPA) and carbachol (CCh) in mouse anococcygeus cells

	$I_{Cl.Ca}$ *(pA)*	$I_{Cl.Ca}$ *duration (s)*	I_{cation} *(pA)*
Caffeine (10 mM)	188 ± 21 (27)	5.2 ± 0.5 (26)	4.8 ± 0.3 (52)
CPA (10 μM)	19.7 ± 2.1 (83)	14.2 ± 0.9 (83)	3.5 ± 0.3 (92)
CCh (50 μM)	238 ± 55 (13)	2.9 ± 0.7 (13)	4.7 ± 0.5 (14)

Cells were held at a membrane potential of −40 mV. The amplitude of the transient calcium-activated chloride current ($I_{Cl.Ca}$) was measured at the peak of the response and its duration at 70% maximum response. The amplitude of the sustained cation current I_{cation} was measured once the inward current reached a steady state. Results are shown as mean ± s.e.mean for the number of observations in parentheses.

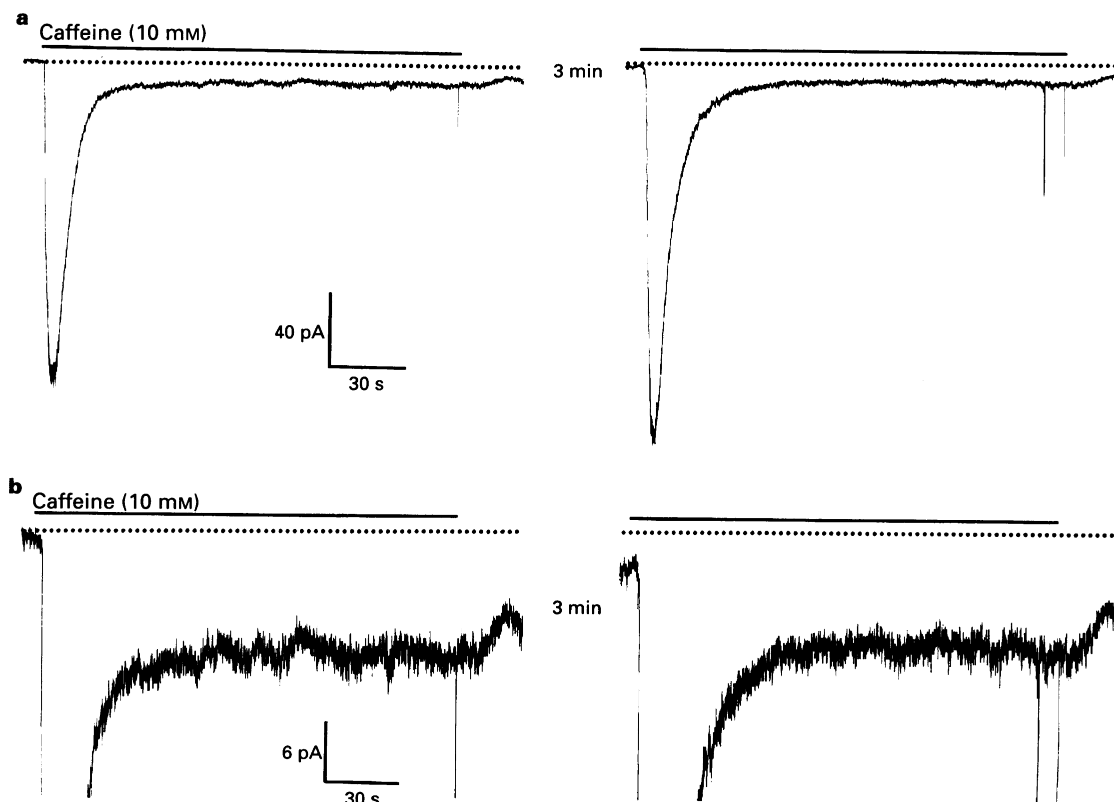

Figure 2 Two consecutive applications of caffeine produce reversible and reproducible responses. (a) Application of caffeine (10 mM for 3 min; shown by the solid bar) produced a large transient inward current followed by a smaller sustained inward current in a mouse anococcygeus cell. Following a 3 min wash, caffeine was applied for a second time and produced a response very similar to the first. (b) Same experiment as (a) with the current shown at a higher gain to highlight the sustained inward current activated by caffeine. In (a) and (b) the holding potential was −40 mV and the dotted line indicates zero current.

Effect of SNP

One of the advantages of caffeine over the other agents used to deplete the calcium stores was that the response to caffeine reversed readily on washing with drug-free solution. Furthermore, a second application of caffeine, applied 3 min after the first, produced both a transient current and a sustained current which did not differ significantly from those produced by the first application (Figure 2, Table 2). Application of SNP following onset of the sustained current produced a rapid reversal of the response to caffeine, inhibiting the sustained current by 86.5±3.0% ($n=30$; Figure 3). Continued application of SNP

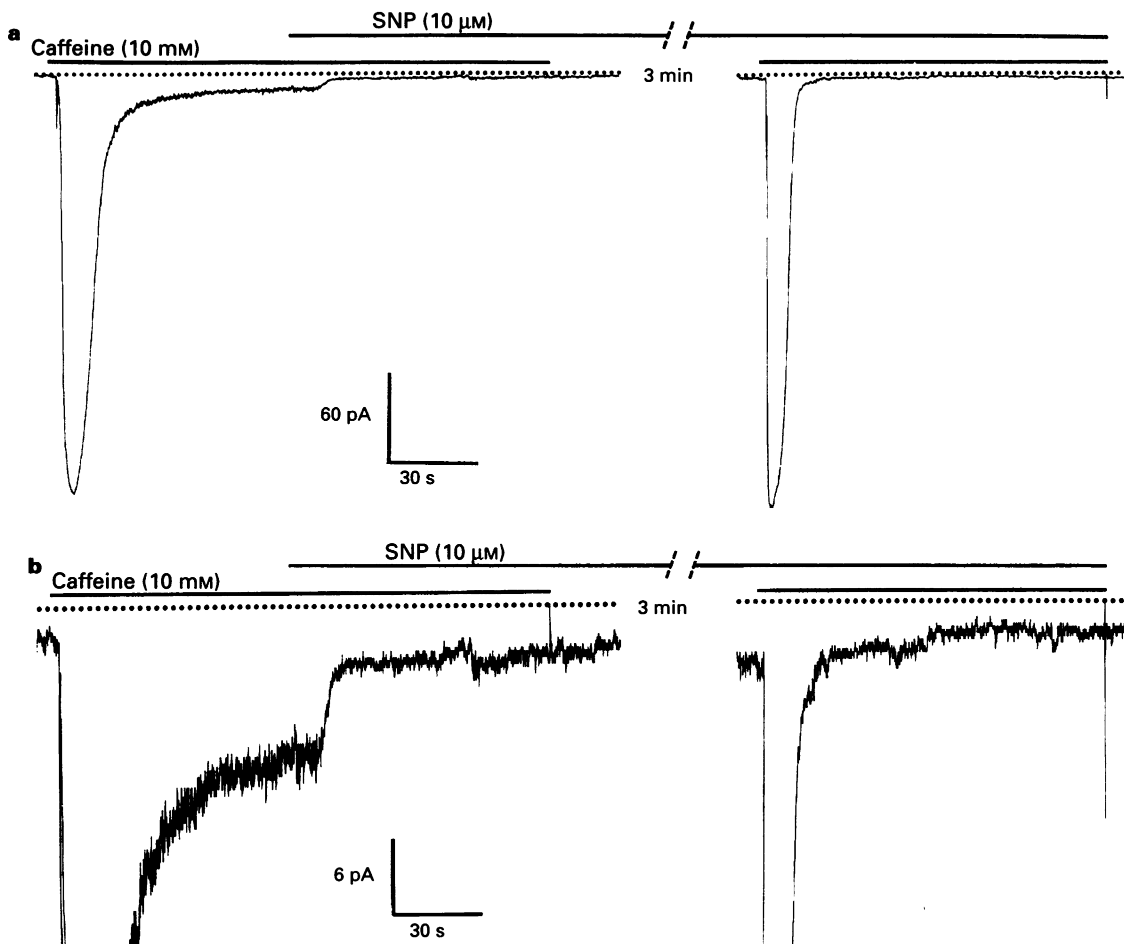

Figure 3 The sustained, but not the transient, component of the caffeine-induced current was blocked by SNP. (a) Caffeine (10 mM) was applied to a mouse anococcygeus cell where indicated by the solid bar, and produced a transient inward current followed by a smaller sustained current. The concomitant addition of sodium nitroprusside (SNP; 10 μM) rapidly inhibited the sustained current. A second application of caffeine following a 3 min wash in caffeine-free solution (SNP present throughout) activated only the transient current, the amplitude of which was similar to that seen in the absence of SNP. (b) Shows the same experiment as in (a) and with the current shown at a higher gain to highlight the effect of SNP on the sustained inward current activated by caffeine. In (a) and (b) the holding potential was −40 mV and the dotted line indicates zero current.

Table 2 The effects of SNP and 8-Br-cGMP, alone or in combination with ODQ, on the electrophysiological response to caffeine in mouse anococcygeus cells

	First application (caffeine alone)		*Second application (caffeine plus drug)*	
	$I_{Cl.Ca}$ *(pA)*	I_{cation} *(pA)*	$I_{Cl.Ca}$ *(pA)*	I_{cation} *(pA)*
Control	186±35 (9)	5.3±0.9 (9)	169±38 (9)	5.2±0.7 (9)
SNP (10 μM)	209±48 (9)	5.9±1.1 (9)	198±49 (9)	Absent (9)
SNP plus ODQ (1 μM)	239±25 (4)	8.3±1.1 (4)	194±35 (4)	8.7±1.3 (4)
8-Br-cGMP(200 μM)	84±14 (4)	4.3±0.3 (4)	74±6 (4)	Absent (4)
8-Br-cGMP plus ODQ (1 μM)	195±25 (4)	8.0±0.4 (4)	145±15 (4)	Absent (4)

Cells were held at a membrane potential of −40 mV. In each case, responses were obtained to two, successive applications of caffeine, each of 3 min duration and separated by a 3 min wash. During the first application, caffeine was applied alone; Sodium nitroprusside (SNP) and 8-Br-cGMP, alone or in combination with the guanylyl cyclase inhibitor ODQ, were applied to the cell during the wash period and the second application of caffeine. In control cells, caffeine was applied alone during both applications. The amplitude of the transient calcium-activated chloride current ($I_{Cl.Ca}$) was measured at the peak of the response. The amplitude of the sustained cation current I_{cation} was measured once the inward current reached a steady state. Results are shown as mean±s.e.mean for the number of observations in parentheses.

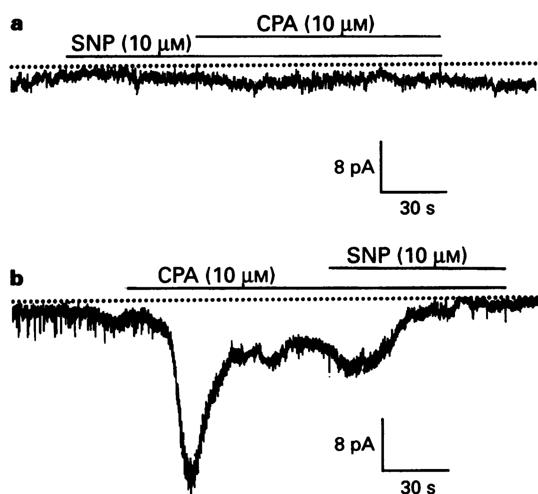

Figure 4 Both the sustained and the transient, components of the CPA-induced current were blocked by SNP. (a) Shows a recording of membrane current from a mouse anococcygeus cell. The application of cyclopiazonic acid (CPA; 10 μM) in the presence (1 min pre-incubation) of sodium nitroprusside (SNP; 10 μM) produced neither a transient nor a sustained inward current; (b) shows the response to CPA (10 μM) in another cell. The concomitant addition of SNP (10 μM) during the sustained inward current produced a rapid inhibition of the CPA effect. In (a) and (b) drugs were applied as indicated by the solid bars, the holding potential was -40 mV and zero current is indicated by the dotted line.

(10 μM) throughout the 3 min wash period and the second application of caffeine did not affect the amplitude of the transient current obtained during the second application of caffeine, but the sustained current was abolished (Figure 3, Table 2).

Unlike caffeine, SNP inhibited both the transient and the sustained components of inward current activated by CPA. The response to CPA was effectively irreversible during the course of a recording so we were unable to obtain more than a single response to the drug in any given cell. We therefore compared the responses to CPA in control cells with those obtained in the presence of SNP. In the presence of the nitrovasodilator, the transient current was absent in 9 out of 11 cells (Figure 4a), whilst in the other 2 cells it was greatly reduced (1 and 2 pA). The sustained current was absent in all 11 cells. Application of SNP (10 μM) following the onset of the response to CPA, reduced the amplitude of the sustained current by $88.6 \pm 3.8\%$ ($n=28$; Figure 4b).

As mentioned above, the electrophysiological response to carbachol was more complex than that to caffeine and CPA since the muscarinic agonist also produced marked oscillations in inward current which appeared to result from activation of calcium-dependent chloride channels. In cells pretreated with SNP (1 μM) the amplitude of the initial transient inward current activated by carbachol (50 μM), was not significantly different from that seen in control cells (209 ± 61 pA; $n=6$). Although not studied in detail, preliminary experiments revealed that the effect of SNP on the carbachol-induced oscillations is complex; in control cells, the oscillations were well-maintained throughout the course of the carbachol application (Figure 1c), whereas in 5 out of 6 cells pretreated with the nitrovasodilator, the oscillations were not sustained (Figure 5b). Application of SNP (10 μM) after the onset of the carbachol-induced oscillations either abolished them completely (4 out of 8 cells; Figure 5a) or produced a marked reduction in their frequency, often accompanied by an increase in amplitude. A more comprehensive study of the effects of SNP on the carbachol-induced oscillations is under way.

Since, in the present study, we were interested primarily in

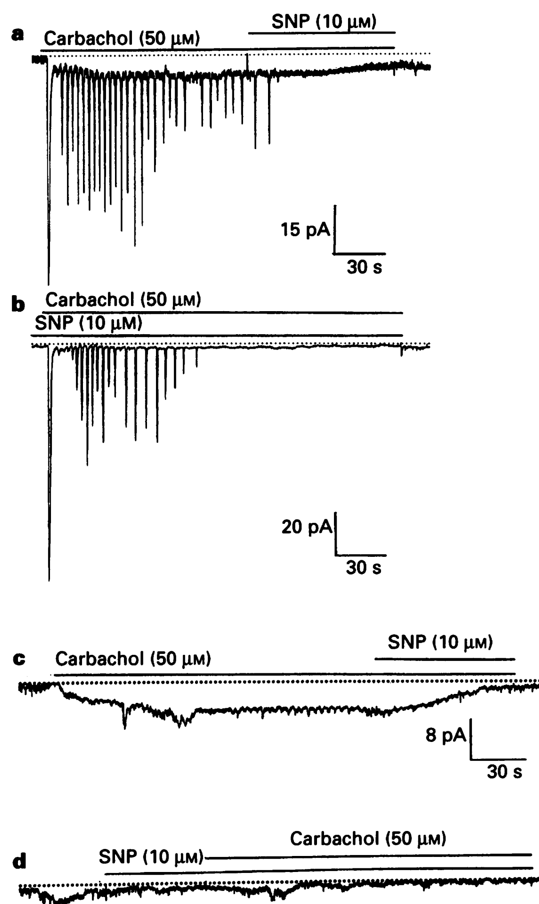

Figure 5 The effect of SNP on the response to carbachol in mouse anococcygeus cells. (a) Application of carbachol (50 μM) evoked a large transient inward current followed by a smaller sustained inward current, superimposed on which, were regular oscillations of inward current. The concomitant application of sodium nitroprusside (SNP; 10 μM) during the sustained current abolished the oscillations and inhibited the sustained current. In (b) carbachol (50 μM) was applied to a cell in the continued presence of SNP (10 μM). The muscarinic agonist evoked a large, transient inward current but no sustained current, and the current oscillations were not well maintained; (c and d) show recordings from two anococcygeus cells bathed in extracellular solution containing the chloride channel blocker, anthracene-9-carboxylic acid (A-9-C; 1 mM). This blocked the large transient current and the oscillations evoked by carbachol and allowed the effects of SNP on the sustained cation current evoked by the muscarinic agonist to be studied in isolation. In (c) application of carbachol evoked a sustained inward current of approximately 5 pA which was inhibited by the concomitant addition of SNP (10 μM). (d) Illustrates the lack of response to carbachol (50 μM) in a cell pre-incubated with SNP (10 μM). In each panel, drugs were applied where indicated by the solid bars, the holding potential was -40 mV and zero current is indicated by the dotted line.

the effects of SNP on the sustained non-selective cation current, all further experiments using carbachol were carried out in the presence of A-9-C at a concentration (1 mM) which we have previously shown not to affect the cation current. Under these conditions, application of carbachol produced only the small sustained current, the initial transient current and the oscillations being inhibited by the chloride channel blocker. Under control conditions the sustained current was present in all cells to which carbachol was applied, and had an amplitude of 6.6 ± 0.7 pA ($n=8$). However, it was absent in cells pretreated with SNP (10 μM; $n=9$; Figure 5d). Application of SNP (10 μM) to a cell following onset of the sustained current quickly reversed the response to the muscarinic agonist, and reduced the amplitude of the sustained current by $88.1 \pm 6.5\%$ ($n=8$; Figure 5c).

Mechanism of action of SNP

Nitric oxide and exogenous nitrates are thought to produce smooth muscle relaxation by activating soluble guanylyl cyclase and thus increasing the intracellular concentration of cyclic GMP. We therefore carried out a series of experiments to determine whether this biochemical pathway was involved in the inhibition of the store-regulated non-selective cation current by SNP. In these experiments caffeine was used throughout to deplete the intracellular calcium stores.

Like SNP, 8-Br-cyclic GMP (200 μM), a membrane-permeable analogue of cyclic GMP, had no effect on the initial transient current activated by caffeine, but inhibited the sustained non-selective cation current (Table 2). As shown in Figure 6, this effect of 8-Br-cyclic GMP was apparent both in cells pretreated with the drug, in which case caffeine activated the transient current but not the sustained current ($n=4$), and when 8-Br-cyclic GMP was applied to cells following onset of the sustained current, in which case the nucleotide reduced the amplitude of the current by $95.0\pm3.0\%$ ($n=5$).

In cells treated with the selective inhibitor of NO-sensitive guanylyl cyclase, ODQ (Garthwaite *et al.*, 1995; 1 μM), SNP (10 μM) no longer inhibited the sustained current activated by caffeine (Table 2). Thus the sustained current was present in cells pretreated with a combination of SNP and ODQ. Furthermore, in cells pretreated with ODQ alone, SNP was nolonger able to inhibit the sustained current when the nitrate was applied after the onset of the caffeine response (Figure 7).

ODQ had no effect on the ability of 8-Br-cyclic GMP to inhibit the caffeine-induced cation current (Figure 7). Interestingly, ODQ alone appeared to increase the size of the cation current; in 4 cells, application of ODQ (1 μM) during the course of the sustained response to caffeine produced a significant increase in the current from 4.5 ± 0.5 pA to 9.5 ± 1.7 pA. Similarly, in 11 cells pre-incubated with ODQ (1 μM) prior to application of caffeine, the sustained current was 7.8 ± 0.7 pA, a value significantly greater than that seen under control conditions (4.8 ± 0.3 pA, $n=52$). ODQ had no effect on the amplitude of the transient inward current activated by caffeine; in 9 cells pre-incubated with ODQ, the amplitude of the transient current recorded following application of caffeine (10 mM) was 199 ± 24 pA, a value not significantly different to that seen under control conditions (188 ± 21 pA, $n=21$). The effects of ODQ on the inhibition of the cation current produced by SNP and 8-Br-cyclic GMP are summarised in Figure 7c.

Discussion

In the mouse anococcygeus muscle, agonists such as phenylephrine and carbachol elicit powerful contractions which, whilst being effectively abolished in the absence of extracellular calcium, are largely insensitive to nifedipine, suggesting that they are mediated by calcium entry into the cell by a pathway distinct from dihydropyridine-sensitive (L-type) calcium

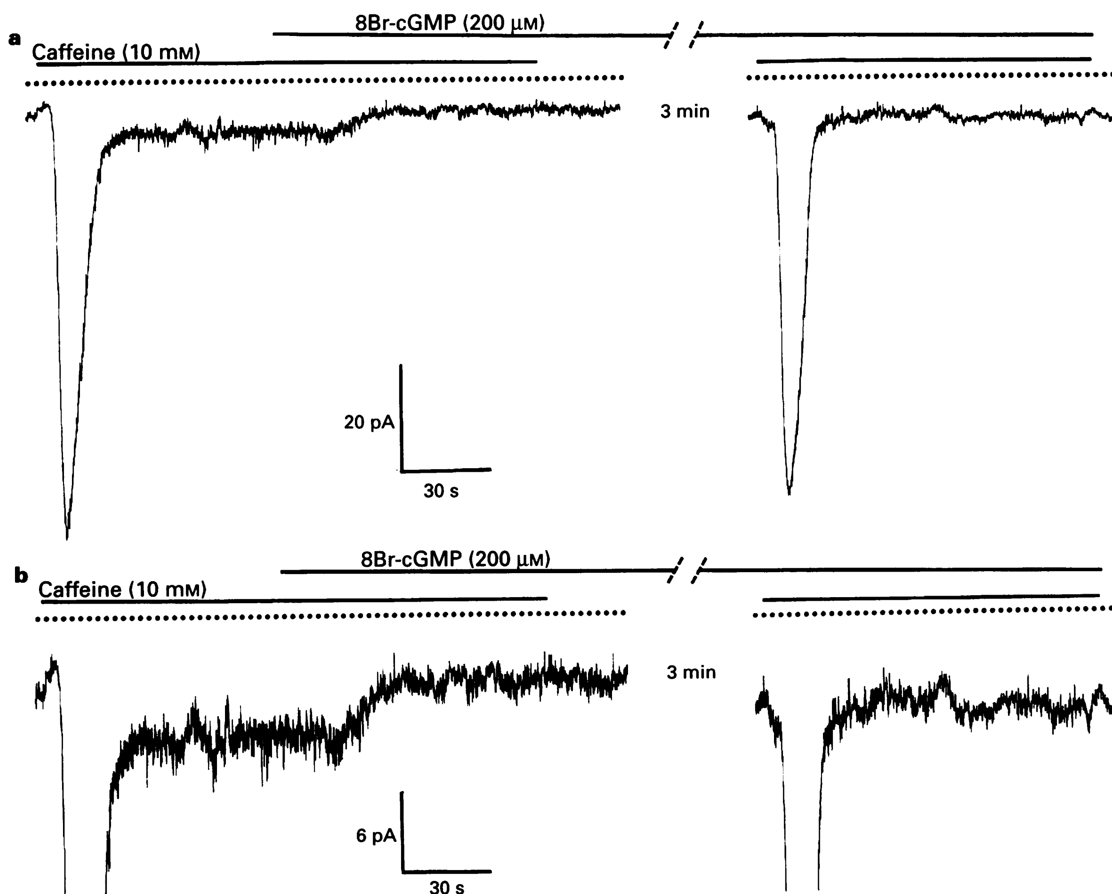

Figure 6 The sustained, but not the transient, component of the caffeine-induced current was blocked by 8-Br-cyclic GMP. (a) Caffeine (10 mM) was applied to a mouse anococcygeus cell where indicated by the solid bar, and produced a transient inward current followed by a smaller sustained current. The concomitant addition of 8-Br-cyclic GMP (200 μM) rapidly inhibited the sustained current. A second application of caffeine following a 3 min wash in caffeine-free solution (8-Br-cyclic GMP present throughout) activated only the transient current, the amplitude of which was similar to that seen in the absence of 8-Br-cyclic GMP. (b) Shows the same experiment as in (a) with the current shown at a higher gain to highlight the effect of 8-Br-cyclic GMP on the sustained inward current activated by caffeine. In (a) and (b) the holding potential was -40 mV and the dotted line indicates zero current.

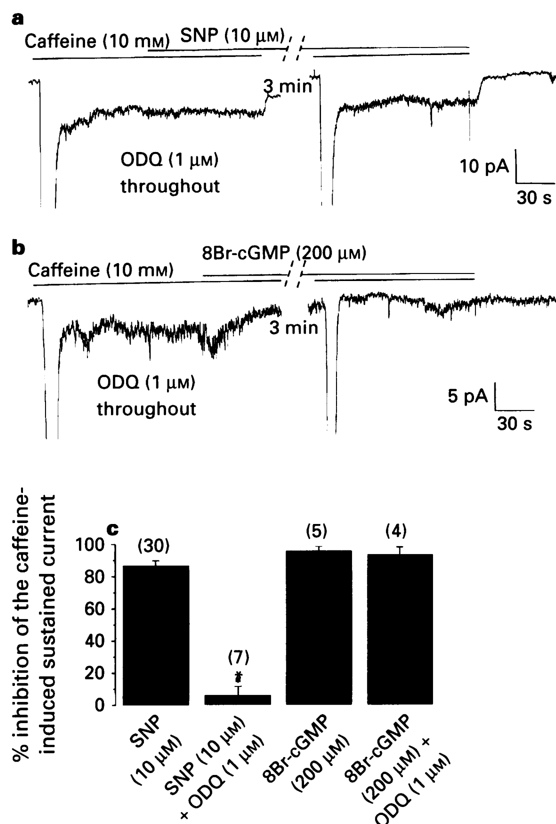

Figure 7 Evidence that sodium nitroprusside (SNP) inhibits the caffeine-induced sustained current by activation of the guanylyl cyclase/cyclic GMP transduction pathway. (a and b) Show recordings from cells bathed in extracellular solution containing the guanylyl cyclase inhibitor, ODQ (1 μM). As shown in (a) ODQ pretreatment inhibited the response to SNP (10 μM), the nitrovasodilator being unable to inhibit the sustained inward current activated by caffeine (cf Figure 3b). In contrast, as shown in (b) ODQ had no effect on the response to 8-Br-cyclic GMP (200 μM), which was able both to reverse the sustained cation current activated by the first application of caffeine and to prevent the activation of the sustained current by a second application of caffeine. The effect of ODQ (1 μM) on the inhibitory actions of SNP (10 μM) and 8-Br-cyclic GMP (200 μM) on the sustained current induced by caffeine (10 mM) are summarised in (c). Bars represent the mean percentage inhibition of the current $\pm$ s.e.mean for the number of cells indicated. *$P<0.05$ compared with SNP alone. In (a) and (b) the holding potential was -40 mV and drugs were applied where indicated by the solid bars.

channels (Gibson *et al.*, 1994b). Furthermore, agents such as CPA which deplete the SR of calcium in a receptor-independent manner also produce contractions with a similar pharmacology, suggesting that it is depletion of the calcium stores in the case of the agonists by IP_3, that is the trigger for calcium entry rather than receptor activation *per se*. Recently we published details of a non-selective cation current activated as a result of store depletion in mouse anococcygeus cells which has properties consistent with it underlying the store-regulated calcium entry process (Wayman *et al.*, 1996a). Given the fundamental importance of this entry process in maintaining prolonged contractions in this tissue, the current represents a potentially important site at which drugs might act to inhibit such contractions. The major inhibitory neurotransmitter in the mouse anococcygeus is NO, or at least a closely related compound (Gibson *et al.*, 1995), and in the present series of experiments we have shown that an exogenous nitrate, SNP, inhibits the store-regulated cation current at concentrations similar to those which relax the whole muscle (Gibson *et al.*, 1994b).

As we have reported previously, caffeine and CPA produced qualitatively similar responses in mouse anococcygeus cells held at a membrane potential of -40 mV; an initial, relatively large transient inward current, followed by a smaller sustained current. These are produced by activation of a calcium-dependent chloride conductance, as a result of the release of calcium from intracellular stores, and a non-selective cation conductance respectively, and we have suggested that the latter current underlies the calcium entry process activated as a result of calcium store depletion (Wayman *et al.*, 1996a). In the present study we have extended the list of agents which can activate this current to carbachol, which contracts the anococcygeus following activation of M_3 muscarinic receptors (Sideso *et al.*, 1994) coupled to PLC (Gibson *et al.*, 1994a). Carbachol activated both the initial transient current and the cation current, presumably as a result of IP_3-mediated calcium release. In addition, carbachol produced rhythmic oscillations of inward current. These oscillations were absent in cells containing EGTA, had a reversal potential close to chloride equilibrium potential, and were inhibited by the chloride channel blocker A-9-C suggesting that they were caused by activation of calcium-dependent chloride channels. Oscillations of calcium-sensitive currents have been described in a variety of smooth muscles (Komori *et al.*, 1993; Lee & Earm, 1994; Kang *et al.*, 1995; Liu & Farley, 1996) and are thought to reflect directly oscillations in the concentration of free intracellular calcium. SNP inhibited the oscillations, supporting the view that the nitrovasodilators interfere with intracellular calcium mobilisation, although further experiments are required to examine this interaction in more detail.

The most important finding of the present study was that SNP inhibited the non-selective cation conductance activated as a result of intracellular calcium store depletion. This action of SNP was apparent regardless of the agent used to deplete the stores, and appeared to be mediated via the guanylyl cyclase/cyclic GMP pathway, being blocked by ODQ, a selective inhibitor of NO-sensitive guanylyl cyclase (Garthwaite *et al.*, 1995), and mimicked by 8-Br-cyclic GMP. ODQ had no effect on the ability of 8-Br-cyclic GMP to inhibit the current, confirming that ODQ acts at the level of guanylyl cyclase to block the transduction pathways as opposed to at a point distal to the production of cyclic GMP. ODQ applied alone increased the amplitude of the non-selective cation current recorded in response to caffeine. One possible explanation for this is that there is background activation of guanylyl cyclase within the smooth muscle cells which is causing a tonic inhibition of the current, and this is removed by ODQ. This possibility is supported by the observation that in whole-muscle preparations, inhibitors of cyclic GMP-dependent phosphodiesterase, for example M&B 22948, produce relaxations of the pre-contracted muscle (Gibson & Mirzazadeh, 1989), again suggesting a tonic activation of guanylyl cyclase. Whilst in the whole muscle this might be due to a continual release of NO from nitrergic nerves, this is unlikely to be the case in the single smooth muscle cell preparations, both because of the absence of nitrergic nerves and also because the inhibitor of NO synthase, N^{ω}-nitro-L-arginine (L-NOARG) had no effect on the cation current activated by CPA (C.P. Wayman, unpublished observations). This suggests that if there is a tonic activation of guanylyl cyclase it is independent of NO production.

It is not clear at this stage how SNP produces its inhibitory action on the store-regulated cation current. The effect of SNP may be mediated at the level of the ion channel such that an increase in the intracellular concentration of cyclic GMP acts to close the channel, for example by a direct action of the nucleotide on the channel, or by cyclic GMP-dependent kinase mediated phosphorylation of the channel or an associated regulatory protein. Alternatively the nitrate may act to refill the depleted calcium store and in doing so remove the primary stimulus for channel opening. At present we cannot reach any conclusions regarding which, if either, of these possible mechanisms underlies the action of SNP, though preliminary results suggest that SNP is unable to inhibit the non-selective

cation current activated by caffeine under conditions where the calcium stores would be unable to refill i.e. in zero intracellular and extracellular calcium (C.P. Wayman, unpublished observations). Furthermore we have obtained evidence in whole muscle preparations that SNP can increase the amount of calcium stored in the smooth muscle (Gibson *et al.*, 1994b). However, arguing against an effect on calcium store refilling was the finding that SNP could inhibit the cation current regardless of the mechanism used to deplete the stores. Caffeine releases intracellular calcium from the SR by acting at a ryanodine-sensitive receptor-operated calcium channel in the SR membrane whilst CPA inhibits sarco-endoplasmic reticulum calcium-ATPases allowing calcium to leak passively from the store (Seidler *et al.*, 1989; Imaizumi *et al.*, 1992). Whilst the ability of both agents, along with carbachol, to activate calcium entry is in accordance with the capacitative model first proposed by Putney (1986), it is less clear how SNP might be able to overcome the actions of CPA and caffeine and allow the stores to refill. In the case of CPA, but not caffeine or carbachol, SNP inhibited the transient, calcium-activated chloride current suggesting that it may prevent CPA from inhibiting the SR calcium-ATPase and thus releasing calcium from the intracellular stores. Cyclic GMP-dependent kinase has been reported to phosphorylate phospholamban, a small inhibitory protein associated with Ca-ATPase. The phosphorylated phospholamban dissociates from the ATPase and it has been suggested that this mechanism underlies the ability of nitrovasodilators to stimulate calcium uptake into intracellular stores (Cornwell *et al.*, 1991). It is possible that the CPA binding site on the Ca-ATPase is closely associated with phospholamban such that the mycotoxin is unable to inhibit the enzyme when it is in the dissociated state, as when influenced by nitrates.

In conclusion, our results indicate that depletion-operated calcium entry may be a possible target for the relaxation mediated by the nitrergic transmitter in non-vascular smooth muscle. Relaxation would therefore be most pronounced, and nitrates most potent, in smooth muscle cells utilising this pathway, particularly tonic smooth muscle.

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