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RESEARCH PAPER

The NADPH oxidase inhibitor diphenyleneiodonium is also a potent inhibitor of cholinesterases and the internal Ca^{2+} pump

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Background and purpose: Diphenyleneiodonium (DPI) is often used as an NADPH oxidase inhibitor, but is increasingly being found to have unrelated side effects. We investigated its effects on smooth muscle contractions and the related mechanisms.
Experimental approach: We studied isometric contractions in smooth muscle strips from bovine trachea. Cholinesterase activity was measured using a spectrophotometric assay; internal Ca^{2+} pump activity was assessed by Ca^{2+} uptake into smooth muscle microsomes.
Key results: Contractions to acetylcholine were markedly enhanced by DPI (10^{-4} M), whereas those to carbachol (CCh) were not, suggesting a possible inhibition of cholinesterase. DPI markedly suppressed contractions evoked by CCh, KCl and 5-HT, and also unmasked phasic activity in otherwise sustained responses. Direct biochemical assays confirmed that DPI was a potent inhibitor of acetylcholinesterase and butyrylcholinesterase (IC_{50} ~8×10^{-6} M and 6×10^{-7} M, respectively), following a readily reversible, mixed non-competitive type of inhibition. The inhibitory effects of DPI on CCh contractions were not mimicked by another NADPH oxidase inhibitor (apocynin), nor the Src inhibitors PP1 or PP2, ruling out an action through the NADPH oxidase signalling pathway. Several features of the DPI-mediated suppression of agonist-evoked responses (i.e. suppression of peak magnitudes and unmasking of phasic activity) are similar to those of cyclopiazonic acid, an inhibitor of the internal Ca^{2+} pump. Direct measurement of microsomal Ca^{2+} uptake revealed that DPI modestly inhibits the internal Ca^{2+} pump.
Conclusions and implications: DPI inhibits cholinesterase activity and the internal Ca^{2+} pump in tracheal smooth muscle.
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Keywords: airway smooth muscle; contraction; NADPH oxidase; cholinesterase; SERCA

Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine iodide; ASM, airway smooth muscle; BChE, butyrylcholinesterase; BTCh, *S*-butyrylthiocholine iodide; CCh, carbachol; CPA, cyclopiazonic acid; DPI, diphenyleneiodonium; L-NNA, *N*-ω-nitro-L-arginine; ODQ, 1H-(1,2,4)oxadiazole(4,3-α)quinoxaline-1-one; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (internal Ca^{2+} pump); TSM, tracheal smooth muscle

Introduction

The primary function of smooth muscle is the conversion of biochemical energy into physical tension, leading to forceful cell shortening. The central mechanism in this energy conversion is the interaction between actin and myosin filaments (the 'sliding filament theory') powered by the ATPase activity of myosin and triggered by phosphorylation of myosin by myosin light chain kinase. However, an increasing number and variety of other signalling events are being found to modulate this excitation–contraction coupling. One recently identified player is c-src (Wijetunge and Hughes 1996; 2007; Ibitayo *et al.*, 1998; Ishida *et al.*, 1999; Brandt *et al.*, 2002; Tolloczko *et al.*, 2002; Hirshman *et al.*, 2005; Krymskaya *et al.*, 2005).

Src or c-src is a family of proto-oncogenic tyrosine kinases originally discovered in chickens as close relatives of the cancer-forming retroviruses v-src, but which have since been shown to play roles in many other cell types and in many other cellular processes: one of these includes excitation–contraction coupling in smooth muscle. In rat tracheal smooth muscle (TSM), Src modulates serotonin-evoked Ca^{2+} signalling (by regulating phosphatidylinositol-3,4-bisphosphate levels and Ca^{2+} influx) and contraction (Tolloczko *et al.*, 2002). In human airway smooth muscle cells, Src mediates thromboxane- (Suzuki *et al.*, 2004) and leukotriene D_4- (Ravasi *et al.*, 2006) induced DNA synthesis and cell proliferation. In guinea pig airway smooth muscle, Src may

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contribute to airway hyper-responsiveness via a mechanism involving increased Ca^{2+} sensitization (Oguma *et al.*, 2007). Src appears to be important in excitation–contraction coupling of other non-airway tissues, including gastrointestinal (Ross *et al.*, 2007) and vascular (Wijetunge and Hughes, 2007) smooth muscles.

Upstream from Src is the oxygen radical-producing enzyme, NADPH oxidase. Peroxide-mediated activation of Src has been investigated in pulmonary arterial myocytes, where it contributes to the regulation of $[Ca^{2+}]_i$ and excitation–contraction coupling (Rathore *et al.*, 2008). Superoxide derived from NADPH oxidase up-regulates the expression of phosphodiesterase 5, and thus increases the excitability of human vascular smooth muscle (Muzaffar *et al.*, 2008).

Thus, there is increasing interest in the roles of NADPH oxidase and Src in smooth muscle function, and increasing need for pharmacological tools to study this signalling pathway. One tool which is frequently used for this purpose is diphenyleneiodonium (DPI) (Cross and Jones, 1986). However, DPI inhibits other flavin-containing enzymes, including nitric oxide synthase (Stuehr *et al.*, 1991; Dodd-o *et al.*, 1997), xanthine oxidase (Sanders *et al.*, 1997), P-450 NADPH reductase (Tew, 1993) and mitochondrial respiratory chain complex I (Majander *et al.*, 1994), raising concerns about its selectivity and about interpretation of data collected using this compound. Recently, in our study of the role of NADPH oxidase signalling in bronchodilator responses in airway smooth muscle, we uncovered a mixture of effects of DPI (unpublished observations). Here, we describe these mixed effects and find them to be mediated by cellular targets other than NADPH oxidase, namely, cholinesterases and the internal Ca^{2+} pump in smooth muscle.

Methods

Preparation of isolated tissues

All experimental procedures were approved by the McMaster University Animal Care Committee, the McMaster University Biosafety Committee and the St. Joseph's Healthcare Research Ethics Board, and conform to the guidelines set out by the Canadian Council on Animal Care. Tracheas were obtained from cows (200–500 kg) killed at a local abattoir and transported to the laboratory in ice-cold Krebs buffer [in mM: NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11 (pH 7.4)]. In the laboratory, the epithelium was removed and strips of smooth muscle were excised (~2–3 mm wide, ~10 mm long); these were used immediately or stored in Krebs buffer at 4°C for use up to 48 h later.

Muscle bath technique

The strips of TSM were mounted vertically in organ baths using silk suture (Ethicon 4-O, Ethicon, Somerville, NJ, USA) tied to a Grass FT.03 force transducer (Harvard Apparatus, Holliston, MA, USA) on one end, and to a glass rod which served as an anchor on the other end. These were bathed in Krebs buffer bubbled with 95% O_2/5% CO_2, maintained at 37°C and supplemented with indomethacin (10 μM) and *N*-ω-nitro-L- arginine (L-NNA; 10^{-4} M) in order to block the production of inhibitory prostaglandins and nitric oxide, respectively, which would suppress mechanical responses. Tissues were passively stretched to impose a preload tension of ≈1 g, and isometric changes in tension were digitized (two samples per second) and recorded on-line (DigiMed System Integrator, MicroMed, Louisville, KY, USA) for plotting on the computer. Tissues were equilibrated for 1 h before starting the experiments, during which time they were challenged with 60 mM KCl three times to assess the functional state of each tissue.

In the first set of experiments, tissues were pretreated for 60 min with DPI (10^{-5}, 3×10^{-5} or 10^{-4} M) or DMSO alone, after which the magnitude and time-course of the responses to carbachol (CCh; 3×10^{-5} M) were examined.

In the second set of experiments, tissues were challenged repeatedly at 30 min intervals (10 min exposure, then 20 min wash period) with either 5-HT (10^{-6} M), potassium chloride (60 mM), CCh (10^{-6} M) or acetylcholine (ACh; 10^{-6} M). These challenges were given three times before supplementing the Krebs media with DPI (10^{-4} M). The peak magnitudes of these agonist-evoked contractions were then quantified and compared.

Quantification of cholinesterase activities

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt, Germany) at 412 nm with a modified Ellman assay (Worek *et al.*, 1999; Eyer *et al.*, 2003). The standard assay mixture (3.16 mL) contained 0.45 mM acetyl thiocholine (AChE) or 1.0 mM butyryl thiocholine (BChE) as substrate, and 0.3 mM 5,5′-dithio-bis-2-nitrobenzoic acid as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37°C. Haemoglobin-free human erythrocyte ghosts were used as source of AChE and were prepared according to Dodge *et al.* (1963) with minor modifications (Worek *et al.*, 2002). Isolated human BChE was suspended in phosphate buffer (0.1 M; pH 7.4).

The inhibitory potency of DPI was assessed by incubation of AChE and BChE with 10^{-6} to 10^{-4} M and 10^{-7} to 10^{-4} M DPI, respectively, for 5 min before adding substrate to determine enzyme activity: IC_{50} values were then determined by non-linear regression analysis of the [DPI]-AChE/BChE relationships. For the determination of the inhibition mode, AChE and BChE activities were quantified after adding different substrate concentrations ($2–8 \times 10^{-4}$ M) in the presence of $0–20 \times 10^{-6}$ M and $0–2.5 \times 10^{-6}$ M DPI, respectively. The reversibility of enzyme inhibition by DPI was tested by incubation of AChE and BChE with 8×10^{-5} M DPI followed by extensive dilution (316-fold) and determination of enzyme activity.

Ca^{2+} uptake

A radiometric assay described previously (Grover and Samson, 1997) was used to quantify Ca^{2+} uptake into crude arterial microsomes prepared from porcine coronary arteries obtained from a local abattoir. In brief, pig coronary artery smooth muscle cells were isolated and plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4), 2 mM glutamine, 50 $mg \cdot L^{-1}$ gentamicin, 0.125 $mg \cdot L^{-1}$

amphotericin B and 10% fetal bovine serum, and then allowed to grow to confluence. Cells were passaged by trypsinization (0.25% trypsin, 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free Hanks's balanced salt solution) for 4 min at 37°C and then replating. After the fifth passage, the cells were homogenized, and 25–75 μg protein was added to 150 μL of a $^{45}Ca^{2+}$ uptake medium so that the final composition of the solutions was 30 mM imidazole–HCl (pH 6.8 at 37°C), 100 mM KCl, 1 mM $MgCl_2$, 5 mM sodium azide, 1 mM EGTA, 0.85 mM $CaCl_2$ (plus trace amounts of $^{45}CaCl_2$), 5 mM ATP, 10 mM creatine phosphate, 50–80 U/mL creatine kinase and 5 mM oxalate. The samples were incubated for 30 min at 37°C in the presence or absence of DPI (3×10^{-5} M or 10^{-4} M) or thapsigargin (10^{-6} M) and then filtered through 0.45 μm nitrocellulose filters, and washed three times with a chilled solution containing 8% sucrose, 0.5 mM EGTA and 40 mM imidazole–HCl (pH 7.0). The filters were then placed in vials containing Beckman Ready Safe cocktail, and the amount of radioactivity in the filters was determined with a scintillation counter.

Data analysis

Contractions were expressed as a percentage of the response to 60 mM KCl added during the equilibration period (immediately before onset of the experiment). AChE and BChE activities were expressed as percentage of control activity. Data are reported as mean ± SEM; *n* refers to the number of animals. Statistical comparisons were made using analysis of variance (with Bonferroni *post hoc* test); $P < 0.05$ was considered statistically significant.

Materials

Names of drugs and molecular targets conform to guidelines in Alexander *et al*. (2008). All chemicals were obtained from Sigma Chemical Company and prepared as 10 mM stock solutions, either as aqueous solutions (KCl; ACh; CCh; 5-HT; acetyl thiocholine, butyryl thiocholine), DMSO (DPI) or ethanol (1H-(1,2,4) oxadiazole(4,3-α)quinoxaline-1-one (ODQ); apocynin). Aliquots were then added to the muscle baths; the final bath concentration of solvents did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Results

DPI directly antagonizes excitatory responses

We first investigated the effects of DPI on cholinergic contractions. Following the equilibration period, tissues were pretreated for 60 min with DPI (10^{-5}, 3×10^{-5}, 10^{-4} M or DMSO alone), then challenged with CCh (3×10^{-5} M). Vehicle-treated controls exhibited a brisk and sustained contraction to CCh. At 10^{-4} M, however, DPI had a marked inhibitory effect on CCh-evoked contractions (Figure 1A,B): the latter were markedly reduced in peak magnitude and became highly transient in nature with phasic activity and spike-like oscillations in tone (Figure 1A). At times, DPI alone raised baseline tone on its own, before any challenge with CCh (not shown).

Next, we examined whether this inhibitory effect of DPI was specific to CCh, or also affected excitatory responses to other spasmogens. Tissues were challenged repeatedly at 30 min intervals with 5-HT (10^{-6} M), KCl (60 mM), CCh (10^{-6} M) or ACh (10^{-6} M; washes out more readily than CCh) three times before and four more times after introduction of DPI (10^{-4} M; $n = 6$ for all). Contractions evoked by 5-HT and CCh were relatively quickly reduced in magnitude by DPI, being nearly abolished by the second challenge in the presence of DPI (Figure 1C). KCl-evoked contractions were somewhat more resistant, being reduced only ~50% by the fourth challenge in the presence of DPI (Figure 1C). On the other hand, ACh-evoked responses were immediately and markedly augmented in the presence of DPI (more than doubled), although the magnitude of this augmentation diminished over the course of the assay (Figure 1C).

Does DPI suppress excitatory responses through action on NADPH oxidase or c-src?

Given that DPI is believed to be a powerful inhibitor of NADPH oxidase, which in turn transduces its effects through generation of superoxide and activation of Src, we examined whether the effect of DPI on CCh contractions could be mimicked by the NADPH oxidase inhibitor apocynin (Stolk *et al*., 1994) or the Src inhibitors PP1 or PP2 (Hanke *et al*., 1996).

Tissues were pretreated for 60 min with vehicle ($n = 9$), DPI (10^{-4} M; $n = 8$), apocynin (10^{-4}; $n = 5$), PP1 (10^{-5} M; $n = 6$) or PP2 (10^{-5} M; $n = 6$) before challenging with 3×10^{-7} M CCh. Only DPI had any statistically significant effect on the magnitude of contractions so evoked (Figure 2). We would therefore conclude that the inhibitory effect of DPI on excitatory responses in bovine TSM is not exerted through NADPH oxidase.

Does DPI suppress excitatory responses through activation of guanylate cyclase?

DPI has also been described as an inhibitor of nitric oxide synthase (Dodd-o *et al*., 1997). However, we would note that our data were collected in the presence of 10^{-4} M L-NNA, and therefore, presume that nitric oxide synthase is already fully inhibited. Nonetheless, we compared the effect of DPI with and without the soluble guanylyl cyclase inhibitor, ODQ. Tissues were pretreated with 10^{-4} M ODQ, with or without 10^{-4} M DPI, for 30 min before testing the response to CCh ($n = 5$). ODQ alone did not reduce cholinergic responses (again, in the presence of L-NNA); more importantly, there was no statistically significant difference between the magnitude of cholinergic responses obtained in the presence of DPI alone and those in the presence of DPI plus ODQ (Figure 2).

Does DPI suppress excitatory responses through inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA)?

Our observations that DPI exerts the same effects – modest increase in baseline tone, suppression of peak magnitude of agonist-evoked responses and unmasking of phasic activity and oscillations in what are otherwise sustained contractions

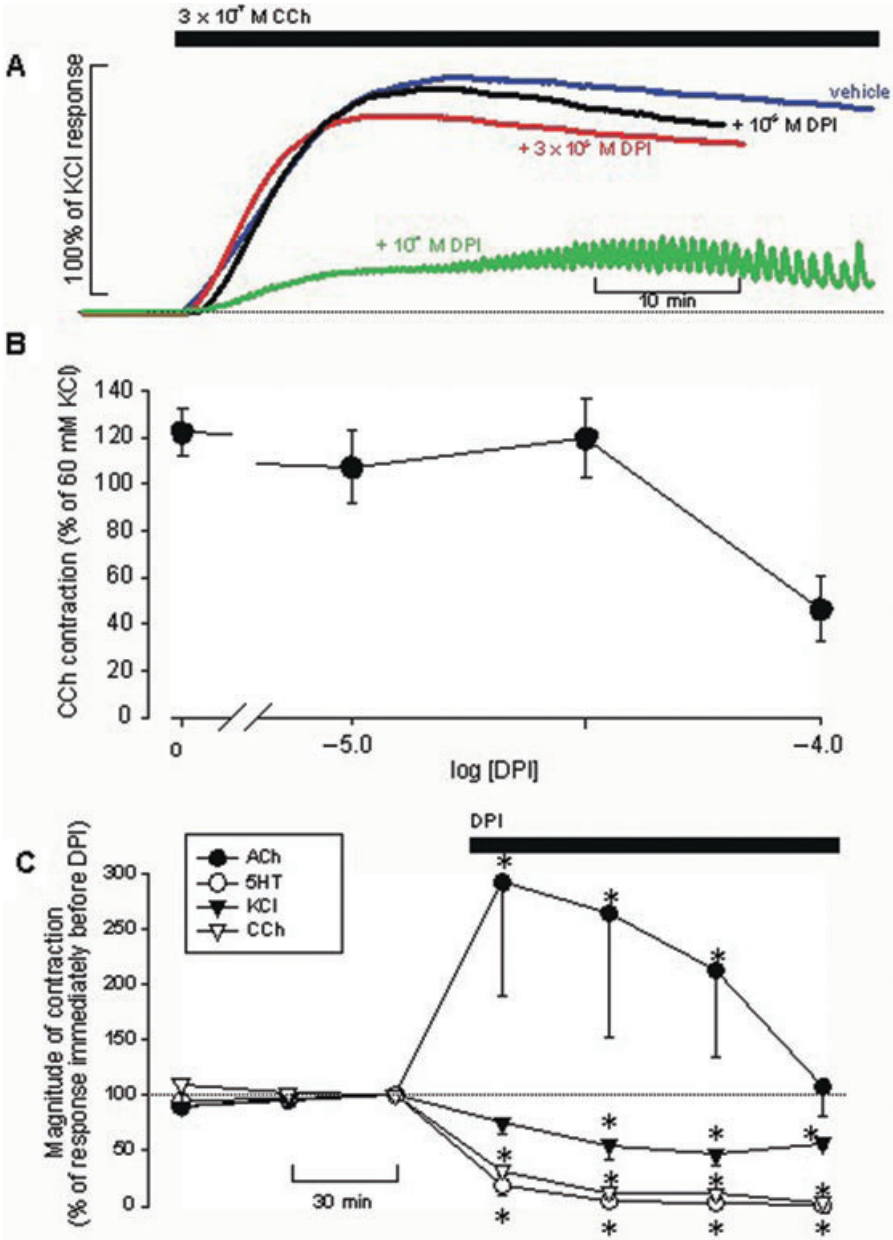

Figure 1 Effects of diphenyleneiodonium (DPI) on mechanical activity in bovine tracheal smooth muscle. (A) Representative tracings showing the increase in tone evoked by 3×10^{-7} M CCh in the absence or presence of DPI (concentrations as indicated); responses are standardized as a % of the response to KCl evoked earlier in the experiment. (B) Concentration–response relationship of the inhibitory effect of DPI on carbachol (CCh)-evoked contractions; symbols indicate mean (±SEM) magnitudes of CCh-evoked responses. (C) Mean (±SEM) responses to four successive additions of acetylcholine (ACh), CCh, 5-HT (all 10^{-6} M) or KCl (60 mM), as indicated, before and after addition of 10^{-4} M DPI to the Krebs solution; $n = 6$ for all. *Significantly different from values before DPI addition.

– as does cyclopiazonic acid, an inhibitor of the SERCA (the internal Ca^{2+} pump) (Janssen *et al.*, 1997; 2001; Helli *et al.*, 2005) – led us to conjecture whether DPI inhibits the internal Ca^{2+} pump. Microsomes were prepared from pig coronary artery ($n = 6$), supplied with ATP (to provide energy to the Ca^{2+} pump) and oxalate (stimulates Ca^{2+} retention in the sarcoplasmic reticulum), and used to evaluate Ca^{2+} uptake in the presence or absence of DPI using previously published methods (Grover and Samson, 1997). A comparison was made with thapsigargin, a well-described SERCA inhibitor (Low *et al.*, 1991). The data from these experiments (summarized in Table 1) confirmed that DPI at the concentrations used in this study partially inhibited SERCA activity.

Does DPI augment ACh-evoked responses through inhibition of AChE?

We also considered the mechanism underlying the transient enhancement of ACh-evoked contractions shown in Figure 1C. Given that ACh responses were augmented, but not those to CCh, we hypothesized that DPI acts by inhibiting activity of AChE. To test this, the activity of human AChE was

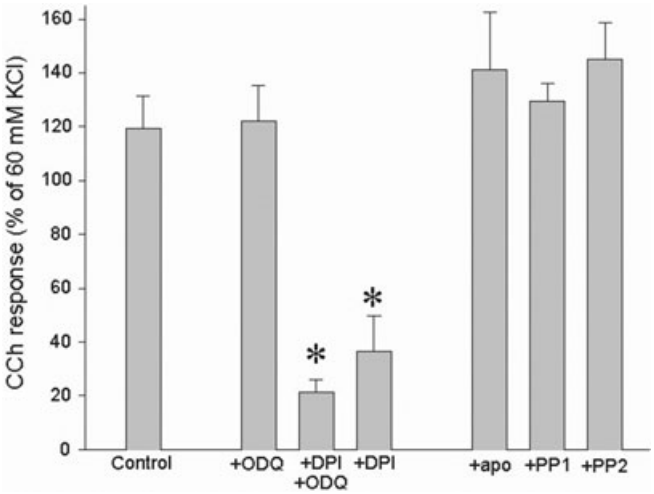

Figure 2 Comparison of effects of various inhibitors on responses to carbachol (CCh). Tissues were pretreated with diphenyleneiodonium (DPI) (10^{-4} M), as well as with ODQ (10^{-4} M; n = 5), apocynin (10^{-4} M; n = 5), PP1 (10^{-5} M; n = 6) or PP2 (10^{-5} M; n = 6) for 30 min before recording the peak contractile response to CCh (3×10^{-7} M). *indicates significantly different from control.

Table 1 Ca^{2+} uptake into microsomes

	Ca^{2+} uptake ($\mu mol \cdot g^{-1}$)	*% Inhibition*
Microsomes alone	45 ± 1.0	NA
Microsomes + thapsigargin (10^{-6}M)	13.2 ± 0.6	71
Microsomes + DPI (3×10^{-5} M)	36.8 ± 0.7	18
Microsomes + DPI (10^{-4} M)	30.5 ± 0.9	32

Microsomes from pig coronary artery were incubated for 30 min in the presence of ATP (5 mM), oxalate (5 mM) and blockers (as indicated), and uptake of $^{45}Ca^{2+}$ was measured.
Values shown in the table are means ± SEM, from six microsomal preparations.
DPI, diphenyleneiodonium.

quantified (see Methods) in the presence of varying concentrations of DPI (10^{-6} to 10^{-4} M); a comparison was made with the effect of DPI (10^{-7} to 10^{-4} M) on the activity of human BChE. DPI was indeed a potent inhibitor of both enzymes, with a concentration for half maximal inhibition (IC_{50}) of 8 μM (95% confidence interval: 6.9–8.6 μM) and 0.6 μM (95% confidence interval: 0.4–0.8 μM), respectively (n = 4 for both; Figure 3). In another experiment, we assessed the nature of this inhibition by measuring enzyme activities over a range of concentrations of substrate (200–800 μM acetyl thiocholine or butyryl thiocholine) and inhibitor (DPI; 0–20 μM) (Figure 4; n = 4): the data indicated a mixed, non-competitive type of inhibition. Finally, we assessed reversibility of this inhibition by incubating AChE or BChE with 80 μM DPI (or solvent), then transferring an aliquot to a cuvette for determination of enzyme activity after dilution (by a factor of 316; n = 4; data not shown) and found that the dilution resulted in a complete recovery of enzyme activity.

Consistent with an inhibitory action of DPI against AChE, we found DPI did not augment ACh-evoked contractions in TSM strips which had already been pretreated for 30 min with

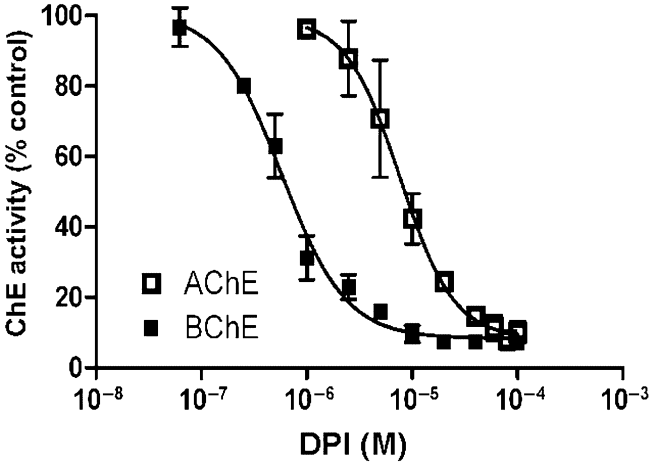

Figure 3 Effect of diphenyleneiodonium (DPI) on cholinesterase activities. The inhibitory potency of DPI was determined by incubation of human AChE and BChE with 10^{-6} to 10^{-4} M and 10^{-7} to 10^{-4} M DPI, respectively, for 5 min before adding substrate to determine enzyme activity. Each point represents the means ± SD of four experiments.

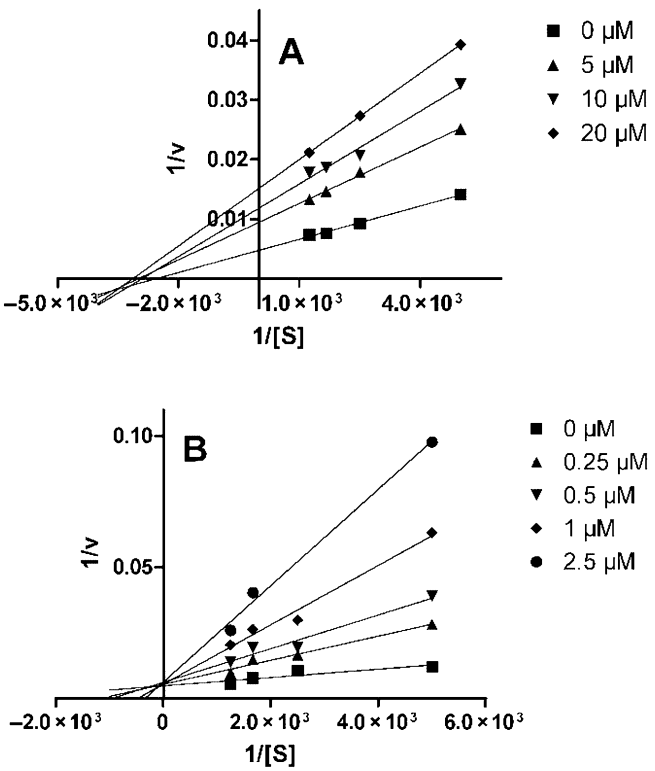

Figure 4 Determination of type of inhibition of cholinesterases by diphenyleneiodonium (DPI). Lineweaver–Burk plots for the inhibition of human AChE (A) and BChE (B) by DPI (concentrations given at right in figure) in the presence of different substrate concentrations (200–800 μM). Data are given as means (n = 4).

the AChE inhibitor neostigmine (10^{-4} M; data not shown); instead, only a gradual reduction in ACh-evoked contractions was seen, much like the changes summarized in Figure 1C for CCh and 5-HT.

Discussion

As outlined above, NADPH oxidase and Src are increasingly found to play important roles in various aspects of cell function, and for this reason pharmacological tools which modulate this signalling pathway are becoming more valuable. However, as with all pharmacological tools, it is important to recognize the limitations of these tools, including their actions on other cellular targets. DPI is no exception. Although often used in the past as a selective NADPH oxidase inhibitor, we here present data which highlight its potent actions against cholinesterases, and less potent actions against SERCA, adding to a long list of actions on other enzymes including nitric oxide synthase (Stuehr *et al*., 1991; Dodd-o *et al*., 1997), xanthine oxidase (Sanders *et al*., 1997), P-450 NADPH reductase (Tew, 1993) and mitochondrial respiratory chain complex I (Majander *et al*., 1994).

The inhibition of AChE (and BChE) was rapid, leading to an immediate augmentation of ACh-evoked contractions, which correlated with the results from the inhibition assays. DPI was shown to be a potent, readily reversible, mixed non-competitive type inhibitor of both human AChE and BChE, with IC_{50} values in the low micromolar (AChE) and high nanomolar range (BChE). At the concentrations used in our muscle bath studies, DPI would have completely inhibited ACh degradation, which explains the marked augmentation of ACh-evoked contractions (but not of CCh-, 5-HT or KCl-evoked contractions).

Although the DPI-induced enhancement of responses to ACh could also be explained by an inhibitory effect on nitric oxide synthase (Stuehr *et al*., 1991; Dodd-o *et al*., 1997; Kesler *et al*., 2002), which otherwise opposes contraction through the synthesis of nitric oxide, our experiments were all performed in the presence of the nitric oxide synthesis inhibitor L-NNA (10^{-4} M), and so this enzyme should already be inhibited. Besides, inhibition of nitric oxide synthase would have effects on all contractor responses, not just those evoked by ACh.

The inhibitory effect on excitation–contraction coupling, on the other hand, was only seen at relatively high concentrations (10^{-4} M, but not 3×10^{-5} M or lower), and shared many characteristics with that of the SERCA inhibitor, cyclopiazonic acid (Amoako *et al*., 1996). First, the effect was not agonist specific, reducing responsiveness to Ach, CCh, 5-HT and KCl. Moreover, the receptor-mediated responses were essentially abolished, while responses to KCl were only partially reduced (contractions to KCl involve voltage-dependent Ca^{2+} influx and activation of Rho-kinase (Janssen *et al*., 2004; Liu *et al*., 2005) rather than release of internal Ca^{2+}). Also, this effect took a considerable amount of time to develop, presumably due to the nature of the underlying mechanism. That is, there is an ongoing 'leak' of Ca^{2+} from the internal store through a number of pathways (ryanodine- and inositol trisphosphate-gated receptor/channels) which is compensated by the action of SERCA: inhibition of the latter leads to unmasking of the leak with transient elevation of $[Ca^{2+}]_i$ (accounting for the transient increase in baseline tone) and gradual depletion of the store (accounting for the concomitant loss of responsiveness). Finally, both cyclopiazonic acid and DPI unmasked phasic activity (Janssen and Sims, 1993; Janssen and Nana, 1997; Janssen *et al*., 1997; 2001). Consistent with our conjecture that DPI was acting to suppress SERCA activity, we did indeed find it to inhibit Ca^{2+} uptake into smooth muscle microsomes. Even though ACh and CCh act on the same cholinergic receptor, the ACh-responses did not appear to decrease to the same level as the CCh responses even by the fourth challenge. It is possible that they would have if we had extended the duration of these experiments, i.e. it may take much longer for this inhibitory effect on SERCA to overcome the enhancement by inhibition of AChE activity). Alternatively, it may be that ACh and CCh couple somewhat differently to the muscarinic receptor in that the former more powerfully activates the RhoA/ROCK pathway, which may explain the additional tone at the end of these experiments.

Overall, we found that DPI – which is often used as an inhibitor of NADPH oxidase – is also a highly potent inhibitor of cholinesterase and SERCA activities. Less germane to the focus of this study, we did not find that either NADPH oxidase or Src contributed to contractor responses in bovine TSM, as the latter were not inhibited by another NADPH oxidase inhibitor (apocynin) nor the Src inhibitors PP1 and PP2. In contrast, Src seems to be important in 5-HT-evoked contraction of rat TSM (Tolloczko *et al*., 2002), as well as ATP-induced airway hyper-responsiveness in guinea pig TSM (Oguma *et al*., 2007). The explanation for the discrepancy between these findings and ours is unclear, but may include species-related differences (i.e. unique to rodents).

Acknowledgements

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References

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC). 3rd edition. *Br J Pharmacol* **153** (Suppl. 2): S1–209.

Amoako D, Qian Y, Kwan CY, Bourreau JP (1996). Probing excitation–contraction coupling in trachealis smooth muscle with the mycotoxin cyclopiazonic acid. *Clin Exp Pharmacol Physiol* **23**: 733–737.

Brandt D, Gimona M, Hillmann M, Haller H, Mischak H (2002). Protein kinase C induces actin reorganization via a src- and rho-dependent. *Pathway J Biol Chem* **277**: 20903–20910.

Cross AR, Jones OT (1986). The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. *Biochem J* **237**: 111–116.

Dodd-o JM, Zheng G, Silverman HS, Lakatta EG, Ziegelstein RC (1997). Endothelium-independent relaxation of aortic rings by the nitric oxide synthase inhibitor diphenyleneiodonium. *Br J Pharmacol* **120**: 857–864.

Dodge JT, Mitchell C, Hanahan DJ (1963). The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* **100**: 119–130.

Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V *et al*.

(2003). Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal Biochem* **312**: 224–227.

Grover AK, Samson SE (1997). Peroxide resistance of ER Ca^{2+} pump in endothelium: implications to coronary artery function. *Am J Physiol* **273**: C1250–C1258.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ *et al*. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* **271**: 695–701.

Helli PB, Pertens E, Janssen LJ (2005). Cyclopiazonic acid activates a Ca^{2+}-permeable, non-selective cation conductance in porcine and bovine tracheal smooth muscle. *J Appl Physiol* **99**: 1759–1768.

Hirshman CA, Zhu D, Pertel T, Panettieri RA, Emala CW (2005). Isoproterenol induces actin depolymerization in human airway smooth muscle cells via activation of an Src kinase and GS. *Am J Physiol Lung Cell Mol Physiol* **288**: L924–L931.

Ibitayo AI, Tsunoda Y, Nozu F, Owyang C, Bitar KN (1998). Src kinase and PI 3-kinase as a transduction pathway in ceramide-induced contraction of colonic smooth muscle. *Am J Physiol* **275**: G705–G711.

Ishida T, Ishida M, Suero J, Takahashi M, Berk BC (1999). Agonist-stimulated cytoskeletal reorganization and signal transduction at focal adhesions in vascular smooth muscle cells require c-Src. *J Clin Invest* **103**: 789–797.

Janssen LJ, Nana R (1997). Na^+/K^+ ATPase mediates rhythmic spontaneous relaxations in canine airway smooth muscle. *Respir Physiol* **108**: 187–194.

Janssen LJ, Sims SM (1993). Emptying and refilling of Ca^{2+} store in tracheal myocytes as indicated by ACh-evoked currents and contraction. *Am J Physiol* **265**: C877–C886.

Janssen LJ, Walters DK, Wattie J (1997). Regulation of $[Ca^{2+}]i$ in canine airway smooth muscle by Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange mechanisms. *Am J Physiol* **273**: L322–L330.

Janssen LJ, Wattie J, Lu-Chao H, Tazzeo T (2001). Muscarinic excitation–contraction coupling mechanisms in tracheal and bronchial smooth muscles. *J Appl Physiol* **91**: 1142–1151.

Janssen LJ, Tazzeo T, Zuo J, Pertens E, Keshavjee S (2004). KCl evokes contraction of airway smooth muscle via activation of RhoA and Rho-kinase. *Am J Physiol Lung Cell Mol Physiol* **287**: L852–L858.

Kesler BS, Mazzone SB, Canning BJ (2002). Nitric oxide-dependent modulation of smooth-muscle tone by airway parasympathetic nerves. *Am J Respir Crit Care Med* **165**: 481–488.

Krymskaya VP, Goncharova EA, Ammit AJ, Lim PN, Goncharov DA, Eszterhas A *et al*. (2005). Src is necessary and sufficient for human airway smooth muscle cell proliferation and migration. *FASEB J* **19**: 428–430.

Liu C, Zuo J, Pertens E, Helli PB, Janssen LJ (2005). Regulation of Rho/ROCK signaling in airway smooth muscle by membrane potential and $[Ca^{2+}]i$. *Am J Physiol Lung Cell Mol Physiol* **289**: L574–L582.

Low AM, Gaspar V, Kwan CY, Darby PJ, Bourreau JP, Daniel EE (1991). Thapsigargin inhibits repletion of phenylephrine-sensitive intracellular Ca^{2+} pool in vascular smooth muscles. *J Pharmacol Exp Ther* **258**: 1105–1113.

Majander A, Finel M, Wikstrom M (1994). Diphenyleneiodonium inhibits reduction of iron–sulfur clusters in the mitochondrial NADH–ubiquinone oxidoreductase (complex I). *J Biol Chem* **269**: 21037–21042.

Muzaffar S, Shukla N, Bond M, Sala-Newby GB, Newby AC, Angelini GD *et al*. (2008). Superoxide from NADPH oxidase upregulates type 5 phosphodiesterase in human vascular smooth muscle cells: inhibition with iloprost and NONOate. *Br J Pharmacol* **155**: 847–856.

Oguma T, Ito S, Kondo M, Makino Y, Shimokata K, Honjo H *et al*. (2007). Roles of P2X receptors and Ca^{2+} sensitization in extracellular adenosine triphosphate-induced hyperresponsiveness in airway smooth muscle. *Clin Exp Allergy* **37**: 893–900.

Rathore R, Zheng YM, Niu CF, Liu QH, Korde A, Ho YS *et al*. (2008). Hypoxia activates NADPH oxidase to increase [ROS]i and $[Ca^{2+}]i$ through the mitochondrial ROS-PKCepsilon signaling axis in pulmonary artery smooth muscle cells. *Free Radic Biol Med* **45**: 1223–1231.

Ravasi S, Citro S, Viviani B, Capra V, Rovati GE (2006). CysLT1 receptor-induced human airway smooth muscle cells proliferation requires ROS generation, EGF receptor transactivation and ERK1/2 phosphorylation. *Respir Res* **7**: 42.

Ross GR, Kang M, Shirwany N, Malykhina AP, Drozd M, Akbarali HI (2007). Nitrotyrosylation of Ca^{2+} channels prevents c-Src kinase regulation of colonic smooth muscle contractility in experimental colitis. *J Pharmacol Exp Ther* **322**: 948–956.

Sanders SA, Eisenthal R, Harrison R (1997). NADH oxidase activity of human xanthine oxidoreductase – generation of superoxide anion. *Eur J Biochem* **245**: 541–548.

Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ (1994). Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* **11**: 95–102.

Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R *et al*. (1991). Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J* **5**: 98–103.

Suzuki Y, Asano K, Shiraishi Y, Oguma T, Shiomi T, Fukunaga K *et al*. (2004). Human bronchial smooth muscle cell proliferation via thromboxane A2 receptor. *Prostaglandins Leukot Essent Fatty Acids* **71**: 375–382.

Tew DG (1993). Inhibition of cytochrome P450 reductase by the diphenyliodonium cation. Kinetic analysis and covalent modifications. *Biochemistry* **32**: 10209–10215.

Tolloczko B, Turkewitsch P, Choudry S, Bisotto S, Fixman ED, Martin JG (2002). Src modulates serotonin-induced calcium signaling by regulating phosphatidylinositol 4,5-bisphosphate. *Am J Physiol Lung Cell Mol Physiol* **282**: L1305–L1313.

Wijetunge S, Hughes AD (1996). Activation of endogenous c-Src or a related tyrosine kinase by intracellular (pY)EEI peptide increases voltage-operated calcium channel currents in rabbit ear artery cells. *FEBS Lett* **399**: 63–66.

Wijetunge S, Hughes AD (2007). Src family tyrosine kinases mediate contraction of rat isolated tail arteries in response to a hyposmotic stimulus. *J Hypertens* **25**: 1871–1878.

Worek F, Mast U, Kiderlen D, Diepold C, Eyer P (1999). Improved determination of acetylcholinesterase activity in human whole blood. *Clin Chim Acta* **288**: 73–90.

Worek F, Reiter G, Eyer P, Szinicz L (2002). Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. *Arch Toxicol* **76**: 523–529.