British Journal of Pharmacology (1998) 124, 911–916
http://www.stockton-press.co.uk/bjp

Selective potentiation by ouabain of naloxone-induced withdrawal contractions of isolated guinea-pig ileum following acute exposure to morphine

[1]M.K. Mundey, R. Mason & V.G. Wilson

School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

1 Ouabain, an inhibitor of Na^+/K^+ ATPase induces the release of acetylcholine from central and myenteric cholinergic neurones principally due to partial depolarization of the cell membrane. The effect of ouabain has been examined on neurogenic contractions in the guinea-pig ileum arising from either electrical field stimulation or from naloxone in morphine-exposed preparations.

2 Guinea-pig isolated ileum preparations were stimulated transmurally (0.1 Hz, 0.3 ms, 200 mA) to elicit contractions of the myenteric plexus-longitudinal smooth muscle.

3 Incubation with morphine (0.3 μM, 60 min) was followed by naloxone (1 μM) which produced withdrawal contractions in 16/26 preparations (median of 10.7 [2.2–40.0]% of a maximal contracture to KCl (60 mM)).

4 In parallel experiments, ouabain (1 μM) was added to the tissue before exposure to morphine (0.3 μM, 60 min). Naloxone (1 μM) subsequently displayed a withdrawal contraction in all 26/26 tissues (57.9 [30.5–151.7]% of a maximal contracture to KCl (60 mM).

5 Ouabain neither affected the concentration-dependent contractions of guinea-pig ileum produced by carbachol nor the inhibition of electrically-evoked contraction produced by morphine (0.3 μM).

6 The muscarinic antagonist atropine (0.1 μM) antagonized control naloxone withdrawal responses. The atropine resistant component, evident in ouabain-treated tissues, was blocked by SR140333((**S**)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]-octane, chloride), a substance P antagonist.

7 Clonidine (α_2-adrenoceptor agonist) inhibited electrically-evoked contractions. Exposure to the α_2-adrenoceptor antagonist RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline), resulted in a contracture which was not significantly enhanced by ouabain (1 μM).

8 Ouabain selectively potentiates the naloxone-induced withdrawal contraction following acute exposure to morphine the major components of which are mediated by both acetylcholine and substance P.

Keywords: Morphine; naloxone; withdrawal-contractions; ouabain; guinea-pig isolated ileum; acetylcholine; substance P

Introduction

The guinea-pig isolated ileum has long been used as a simple model to study the cellular and neurochemical basis for opiate tolerance and dependence. Morphine inhibits both spontaneous and electrically-evoked, cholinergic contractions of the guinea-pig isolated ileum by hyperpolarization of S neurones in the myenteric plexus (Suprenant & North, 1988; Leedham *et al*., 1992). This action is mediated by activation of μ-opioid receptors. Abrupt displacement of morphine from its receptor, by exposure to the μ-opioid receptor antagonist naloxone, caused transient depolarization of the S neurones and a withdrawal-contracture, which is thought to be indicative of the development of dependence (Johnson *et al*., 1987). Pharmacological studies on the withdrawal contractions elicited by naloxone provides evidence for the involvement of at least two neurotransmitters, acetylcholine and a tachykinin, eg. substance P, (Chahl, 1983; Tsou *et al*., 1985), both of which are known to be co-localized in the guinea-pig myenteric plexus (Costa *et al*., 1996) and are released from cholinergic neurones (Furness & Costa, 1987).

High concentrations of ouabain, an inhibitor of Na^+/K^+ ATPase, have been shown to cause the release of acetylcholine from both central (Vizi, 1972; Satoh & Nakazato, 1992) and myenteric (Gomez *et al*., 1996) neurones, which suggests that resting membrane potential of cholinergic neurones is determined by the activity of the Na^+/K^+ electrogenic pump. This accords with the observation that low concentrations of ouabain partially depolarize S neurones of the myenteric plexus of the guinea-pig ileum (Kong *et al*., 1997), and is consistent with the suggestion that inhibition of Na^+/K^+ ATPase causes the opening of N-type Ca^{2+} channels, elevation of intracellular calcium ions, and subsequent release of acetylcholine (Gomez *et al*., 1996). In view of the central role of cholinergic neurones in electrically-evoked and naloxone-induced contractions of the guinea-pig ileum, we have compared the effect of ouabain against both responses. In addition, we have also examined the effect of ouabain against contractions elicited by the muscarinic agonist carbachol, and withdrawal responses elicited by addition of an α_2-adrenoceptor antagonist, RX811059 (2-(-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline) following exposure to the α_2-adrenoceptor agonist clonidine (Chahl, 1983).

Methods

Male Dunkin-Hartley guinea-pigs (500–900 g) were killed by a blow to the head and exsanguination. The ileum was

[1] Author for correspondence.

exteriorized, the ileo-caecal junction located and approximately 70 cm removed. A 10 cm segment of the terminal portion was discarded before the contents in the remaining ileum were flushed out with modified Krebs-Henseleit saline. Four 5 cm ileal segments were set up under 2 g of tension in individual 20 ml organ baths, ready for isometric recording using Grass FT03 transducers connected to CED 1902 (Cambridge Electronic Devices, Cambridge) units for amplification. These were linked via a 1401 interface to a 486-33 MHz PC running Spike 2 software (CED). Preparations were incubated in Krebs-Henseleit saline, gassed (95% O_2/5% CO_2) at 37°C and allowed 30 min to equilibrate. Contractions of the tissue were elicited by transmural stimulation (0.1 Hz, 0.3 ms, 200 mA) and allowed a further 45 min to establish reproducible responses.

Control and ouabain experiments

Once stable contractions to electrical field stimulation were established, the tissue was exposed to 0.3 μM morphine for 60 min after which the stimulator was switched off and 1 μM naloxone added. After the effect of naloxone was observed the stimulator was switched back on and the magnitude of the electrically-evoked contractions noted. In other experiments, 1 μM ouabain was added once basal electrically-evoked contractions were established, before exposure to morphine (0.3 μM), and the same procedure followed. In a second series of experiments, tissue was incubated with 0.1 μM clonidine for 60 min after which the incidence of withdrawal contracture to the α_2-adrenoceptor antagonist RX811059 (1 μM) (Mallard *et al*., 1992) was examined. These responses were also tested following preincubation with 1 μM ouabain. A parallel set of experiments were carried out on preparations incubated with morphine followed by naloxone and also in the presence of ouabain (as described above), to allow a direct comparison of the effect of ouabain on the α_2-adrenoceptor and μ-opioid receptor systems.

Effect of atropine and SR140333

The muscarinic antagonist atropine (0.1 μM) and the substance-P antagonist SR140333 (1 μM) were used to determine the transmitter responsible for the naloxone contraction in control and ouabain-treated ileum. The drugs were added 30 min after morphine either individually or together; 30 min was allowed before naloxone (1 μM) was applied.

Ouabain evaluated against carbachol concentration-response curves

Non-cumulative concentration-response curves (CRC) to carbachol were constructed by exposing the preparations to incremental concentrations (2 nM – 1 μM) for 2 – 3 min. Each preparation was then washed twice over 2 min and allowed a further 10 min to recover. Ouabain (1 μM) was added 10 min before each addition of carbachol. At the end of each experiment the preparations were exposed to 60 mM KCl to elicit a maximal contracture of the tissue, a standard against which all responses were related.

Analysis of data

Unless stated otherwise, withdrawal responses have been calculated as a percentage of the contraction to 60 mM KCl and are shown as the median along with the range. The effect of morphine and clonidine on electrically-evoked contractions, in the presence and absence of the antagonist, has been expressed as a percentage of the control response and shown as the mean $\pm$ s.e.mean. Differences between groups have been compared using a Mann-Whitney 2-tailed test and considered statistically significant if $P<0.01$. The potency of carbachol in the absence and presence of ouabain was assessed as the negative logarithm of the concentration required to cause 50% of the maximum response (pD_2) using the logistic equation described by DeLean *et al*. (1978) with Kaleidagraph software (Synergy) on a Macintosh LC II computer. (n represents the number of animals).

Drugs

The comparison of the modified Krebs-Henseleit saline was (mM): NaCl 118, KCl 4.8, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25 and glucose 11.1. Morphine sulphate was obtained from the Pharmacy Department, Queens Medical Centre, ouabain, naloxone hydrochloride, atropine sulphate, clonidine HCl, RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline) from Sigma and SR140333 ((**S**)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2] octane, chloride) from Sanofi (France). All drugs were dissolved in distilled water and added in a volume less than 0.1% of the organ bath volume.

Results

Electrical stimulation of the guinea-pig ileum (0.1 Hz) produced transient contractions (1.98 $\pm$ 0.17 g wt.) that were approximately 60% of the response to 60 mM KCl (3.17 $\pm$ 0.28 g wt., $n=26$).

Figure 1 shows the exposure to 1 μM ouabain caused a transient contraction (15 – 20 min duration) of the ileum; this effect was observed in 15 of 26 preparations, while in the remaining preparations ouabain failed to elicit a response. The basal electrically-evoked contractions (2.33 $\pm$ 0.19 g wt., $n=26$) were not substantially altered following exposure to 1 μM ouabain (2.17 $\pm$ 0.16 g wt., $n=26$). Morphine (0.3 μM) produced similar inhibition of electrically-evoked contractions in the absence (5.4 $\pm$ 1.5% ($n=26$) of control electrically-evoked responses) and in the presence of 1 μM ouabain (8.0 $\pm$ 1.5% ($n=26$) of control electrically-evoked contractions).

Naloxone (1 μM) caused a transient, withdrawal-contraction in 16/26 preparations exposed to 0.3 μM morphine, whereas in the corresponding ouabain-treated tissues all 26 preparations responded on application of the antagonist (Figure 2). In the absence of morphine, naloxone failed to elicit a contraction either in the absence ($n=5$) or presence ($n=5$) of 1 μM ouabain. In control preparations (0.3 μM morphine alone), naloxone-induced withdrawal contractions ranged from 2.2 to 40.0% of the response to 60 mM KCl (median - 10.7%, $n=16$), while in the presence of 1 μM ouabain (and 0.3 μM morphine) there was a 5 fold enhancement of the contractions (median - 57.9%, $n=26$) ranging from 36.0 $\pm$ 151.7% of the response to 60 mM KCl (Figure 2). As shown in Figure 3, naloxone-induced contractions in the presence of ouabain often comprised two components, a rapid response reaching a peak within 20 s and a slower response lasting 4 – 5 min.

Electrically-evoked contractions, elicited once the withdrawal responses had waned to baseline (see Figure 3), were similar in the absence (82.0 $\pm$ 8.3% of the control value, $n=26$)

and presence of 1 μM ouabain (73.1 $\pm$ 6.6% of the control value, $n=26$); naloxone caused a similar reversal of morphine-induced inhibition of electrically-evoked contractions even in the 10/26 control preparations that failed to respond with a withdrawal contraction. In another sequence of experiments, carbachol produced concentration-dependent contractions of the guinea-pig isolated ileum (pD_2 - 7.22 $\pm$ 0.10, E_{max} - 97.0 $\pm$ 3.2, of 60 mM KCl, $n=7$). Ouabain (1 μM) failed to affect either the potency (pD_2 - 7.18 $\pm$ 0.14), or the maximum response (E_{max} - 94.7 $\pm$ 7.4, $n=7$) to carbachol.

As shown in Figure 4, atropine (0.1 μM) practically abolished the naloxone-induced withdrawal responses (25 [3.1 – 56.9]%, $n=11$) in control preparations. However, in the presence of 1 μM ouabain the withdrawal response to naloxone (76.9 [30.5 – 151.7]%, $n=11$) was significantly reduced (23.9 [6.0 – 56.7]%, $P<0.01$, $n=11$), leaving a sizeable, atropine-insensitive component of the response. In further experiments, the combination of 0.1 μM atropine and 1 μM SR140333 abolished naloxone-induced withdrawal response in the presence of 1 μM ouabain (Figure 4).

A separate series of experiments was carried out to investigate whether α_2-adrenoceptors, which also inhibit electrically-evoked contractions of the guinea-pig ileum (Malta *et al*., 1981), are influenced in a similar manner to μ-opiate receptors. Clonidine (0.1 μM) inhibited the electrically-evoked contractions to 9.4 $\pm$ 2.1% ($n=7$) of the basal responses and on application of the α_2-adrenoceptor antagonist RX811059 (1 μM) the tissues underwent a withdrawal contraction of 26.0 [9.5 – 68.1]% of the response to 60 mM KCl ($n=7$). When corresponding preparations were preincubated with 1 μM ouabain, the median withdrawal contraction increased to 52.3 [6.0 – 83.3]% ($n=7$) (Figure 5), but this did not reach statistical significance. The electrically-evoked contractions which followed the withdrawal contracture returned to 80.1 $\pm$ 5.7% of the control-evoked contractions, suggesting that clonidine inhibition was overcome by RX811059. Associated morphine exposed preparations gave a naloxone contracture of 19.8 [4.2 – 87.8]% of the KCl response which in the presence of 1 μM ouabain increased significantly to 75.8 [44.5 – 100]% ($P<0.01$, $n=7$).

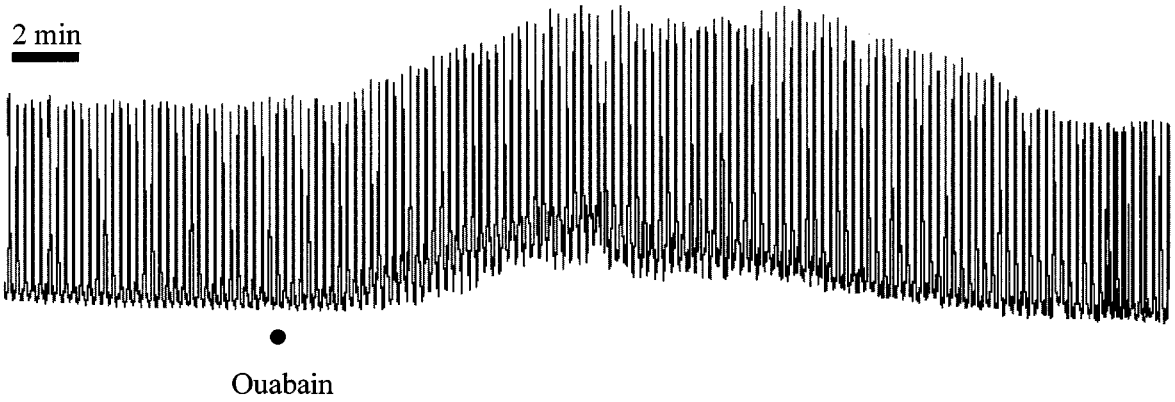

Figure 1 Representative trace recording of the effect of 1 μM ouabain on the guinea-pig isolated ileum stimulated by electrical pulses (0.1 Hz, 1 ms, 200 mA). Note that ouabain produced a transient contraction without significantly altering the electrically-evoked contraction.

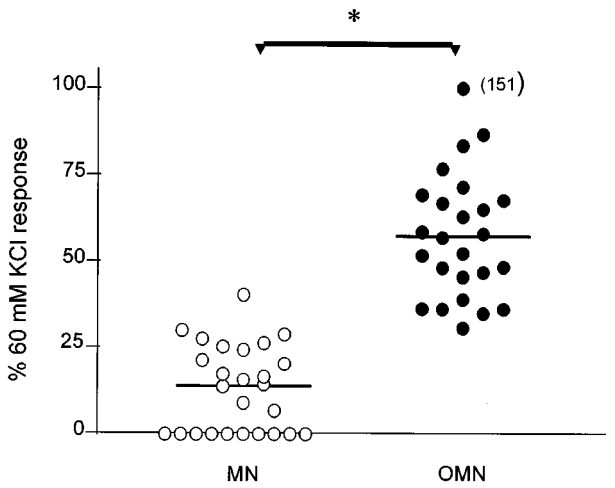

Figure 2 Scatterdiagram of naloxone (N; 1 μM)-induced contractions of morphine (M; 0.3 μM)-exposed segments of the guinea-pig ileum in the absence (MN) and presence (OMN) of 1 μM ouabain (O). Ouabain caused a significant potentiation of the withdrawal contraction (*$P<0.01$, $n=26$). The horizontal line represents the median contraction of the preparations under the two conditions (151 is an outlying value for the OMN group).

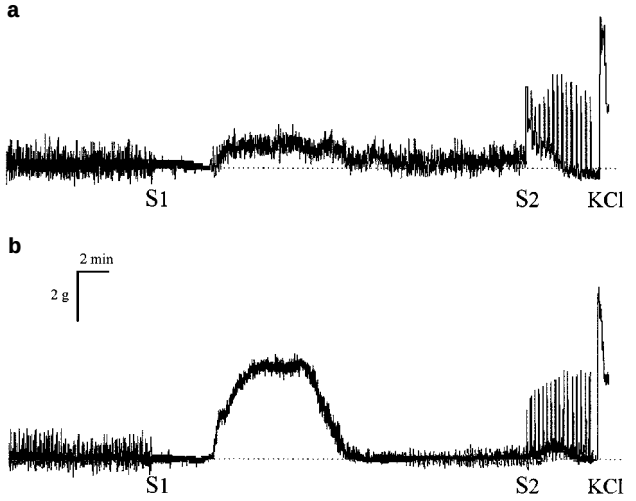

Figure 3 Representative trace recording of the effect of 1 μM naloxone on morphine (0.3 μM)-exposed segments of guinea-pig ileum in the absence (a) and presence (b) of 1 μM ouabain. Morphine was present for 60 min before the addition of naloxone. S_1 and S_2 indicate where the stimulator was turned off and on, respectively. KCl (60 mM) was added after stable, electrically-evoked contractions had been established.

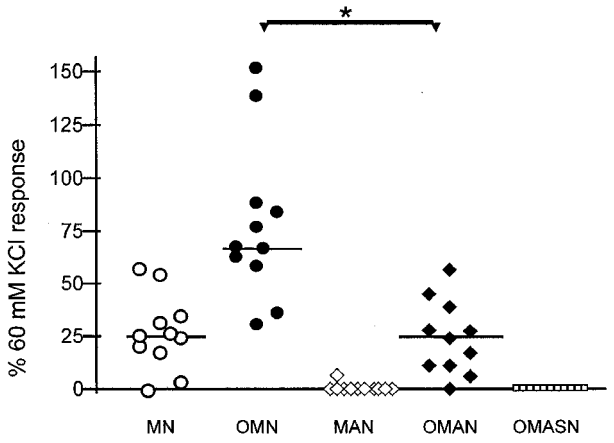

Figure 4 Naloxone (N; 1 μM)-induced contractions of morphine (M; 0.3 μM)-exposed segments of the guinea-pig ileum in the absence (MN) and presence of 1 μM ouabain (OMN). Atropine (A; 0.1 μM) inhibited the control withdrawal contractions (MAN). Also shown are the responses in the presence of either 0.1 μM atropine (OMAN) or a combination of 0.1 μM atropine and 1 μM SR-140333 (OMASN). Responses have been expressed as a percentage of the contractions to 60 mM KCl and are shown as the mean $\pm$ s.e.mean of 11 observations. The horizontal line represents the median contraction. *Indicates a statistically significant difference ($P<0.01$) between the responses in the presence of 1 μM ouabain alone (OMN) and 1 μM ouabain plus 0.1 μM atropine (OMAN).

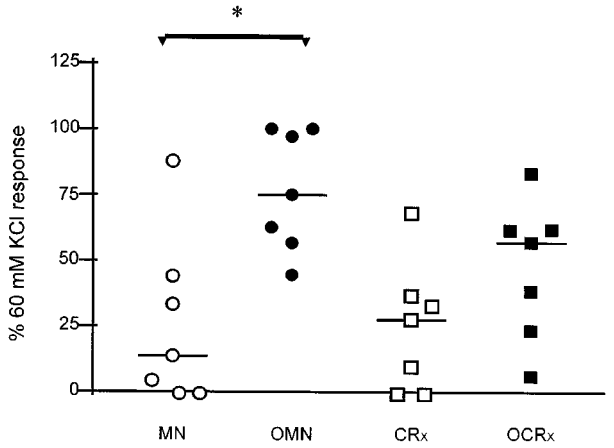

Figure 5 Scatterdiagram of naloxone (N; 1 μM)-induced contractions of morphine (M; 0.3 μM)-exposed segments of the guinea-pig ileum in the absence (MN) and presence (OMN) of 1 μM ouabain (O). Also shown are RX811059 (RX; 1 μM)-induced contractions of clonidine (C; 0.1 μM) exposed segments in the absence (CRx) and presence (OCRx) of ouabain (1 μM). The horizontal line represents the median contraction of 7 observations. Ouabain pretreatment did not significantly increase the contracture to RX811059 (1 μM) as it did for corresponding naloxone contractures in the morphine exposed preparations (*$P<0.01$).

Discussion

In the present study ouabain produced relatively minor effects on contractions elicited either by electrical stimulation of cholinergic nerves or by carbachol, responses mediated by muscarinic receptors (Barlow *et al.*, 1976; Benham *et al.*, 1985). The transient, but variable, effect on the electrically-evoked contractions is not consistent with the reported ability of ouabain to stimulate the release of acetylcholine from the guinea-pig ileum, although this was most pronounced with much higher concentrations (100 μM; Gomez *et al.*, 1996). However, the above findings are entirely consistent with the recent report by Kong and co-workers (1997) that exposure of S neurones to ouabain was associated with either depolarization, no change in membrane potential, or, in a few instances, hyperpolarization. In addition, ouabain did not affect either morphine-induced inhibition of electrically-evoked contractions or the subsequent ability of naloxone to overcome the effect of morphine — at least as judged by the magnitude of the electrically-evoked contractions.

The most striking observation of the present study is that ouabain caused a pronounced increase in both the frequency (to 100%) and magnitude (5 fold) of the withdrawal contractions elicited by naloxone in morphine-exposed segments of the guinea-pig isolated ileum. Pharmacological data indicate that this effect was associated with a change in the transmitter(s) contributing to the response. In the absence of ouabain, the muscarinic receptor antagonist atropine abolished the withdrawal contraction to naloxone, indicating that acetylcholine was the sole contributor. In contrast, a sizeable atropine-resistant component (approximately 1/3rd) of the response to naloxone was noted in the presence of ouabain. Since no response was observed in the presence of a combination of atropine (0.1 μM) and SR 140333 (1 μM), a NK_1 receptor antagonist (Emonds-Alt *et al.*, 1993), the available evidence suggests that both acetylcholine and substance P are implicated in the response in the presence of ouabain. Although the latter conclusion is similar to that of earlier studies not involving ouabain (Chahl, 1983; Tsou *et al.*, 1985), this is the first study to suggest a role of Na^+/K^+ ATPase in limiting the magnitude and frequency of naloxone-induced withdrawal contractions in the guinea-pig ileum.

Na^+/K^+ ATPase is present on both myenteric nerves and the associated ileal smooth muscle (Johnson & Fleming, 1989) and is thought to contribute to the resting membrane potential or act to limit the magnitude of changes following membrane depolarization (Kong *et al.*, 1997). However, since ouabain failed to alter carbachol-induced contractions, a response known to involve depolarization of the smooth muscle (Bolton, 1976; Lim & Bolton, 1988), it seems likely that the enhancement of naloxone-induced contractions is at the level of the neurones; the failure of ouabain to influence contractions to single pulses (0.1 Hz) may be due to the very brief period of neuronal depolarization which is too short for significant activation of Na^+/K^+ ATPase.

Acetylcholine and substance P are known to be co-localized in guinea-pig myenteric neurones but, depending on the frequency of electrical stimulation, make differing contributions to the associated motor response. For example, Galligan (1993) has shown that while low frequencies of electrical stimulation are entirely cholinergic, substance P is involved in responses of longer duration. In the absence of ouabain, therefore, depolarization of S type neurones, arising from the abrupt removal of morphine from opioid receptors by naloxone (Johnson *et al.*, 1987), could be limited by simultaneous activation of the Na^+/K^+ ATPase pump which in turn would reduce the amount of acetylcholine released. However, following inhibition of the Na^+/K^+ ATPase, naloxone-induced depolarization would be of greater magnitude, and/or duration, and result in the release of greater amounts of both acetylcholine and substance P. The resulting contraction of the ileum, arising from the simultaneous activation of muscarinic and NK_1 receptors, is also likely to reflect the ability of these two receptors to interact in a synergistic manner (Holzer & Maggi, 1994). Such a model could account for both the failure of naloxone to elicit a

withdrawal contraction even in preparations where there is conclusive evidence that the inhibitory effect of morphine has been overcome (as judged by the electrically-evoked contractions), and also the ability of ouabain to uncover/enhance these responses. Direct support for this proposal would be provided by direct measurement of the membrane potential of S type myenteric neurones (see: Johnson *et al*., 1987).

Previous studies demonstrated that clonidine was able to block the withdrawal contraction of opiate-dependent guinea-pig ileum elicited by naloxone (Collier *et al*., 1981; David *et al*., 1993). This is principally through inhibition of the release of acetylcholine via presynaptic α_2-adrenoceptors on cholinergic neurones (Alfaro *et al*., 1990) — a neural rather than a muscular site of action which warranted further investigation using ouabain. The present work has shown that clonidine inhibits the acetylcholine-mediated electrically-evoked contractions to an extent comparable to that produced by morphine. In the presence of clonidine, RX811059, a selective α_2-adrenoceptor antagonist (Mallard *et al*., 1992), caused withdrawal contractions of similar magnitude to those elicited by naloxone in morphine-exposed preparations. Ouabain increased the median size of the contractions to RX811059 by almost two fold, but this did not reach significance. In contrast, parallel experiments with naloxone and morphine once again showed that ouabain enhanced the withdrawal response significantly, by four fold. Thus, ouabain selectively enhances naloxone-induced withdrawal contractions of the morphine exposed guinea-pig isolated ileum.

Since high concentrations of ouabain also stimulate the release of neurotransmitter from central neurones (Vizi, 1972; Satoh & Nakazato, 1992), the possibility exists that Na^+/K^+ ATPase may also contribute to resting membrane potential in the brain. We have recently shown that naloxone causes an increase in firing rate of morphine-exposed slices of the hamster (Cutler & Mason, 1995) and rat (Mundey *et al*., 1997) suprachiasmatic nucleus. This effect was only observed in approximately 30% of preparations and bears striking similarities to the observations in the guinea-pig isolated ileum, but differed from that observed in the slices of rat locus coeruleus where naloxone simply reversed the effect of morphine on firing rate (Mundey *et al*., 1997). Future studies will explore the possibility of major differences in the contribution made by Na^+/K^+ ATPase to the electrophysiological characteristics of neurones in the two regions.

In summary, we have demonstrated that ouabain significantly enhances the withdrawal contractions of the guinea-pig ileum exposed to morphine. The pharmacological data presented herein suggest that this may be due to prolonged depolarization of S type myenteric neurones and subsequent release of both acetylcholine and substance P.

M.K.M. is supported by the BBSRC (Realising Our Potential Award).

References

ALFARO, M.J., COLADO, M.I., LOPEZ, L. & MARTIN, M.I. (1990). Effect of clonidine, nimodipine and diltiazem on the *in vitro* opioid withdrawal response in the guinea-pig ileum. *Br. J. Pharmacol.,* **101,** 958–960.

BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SIOH, K.S. (1976). A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in the guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C. *Br. J. Pharmacol.,* **58,** 613–620.

BENHAM, C.D., BOLTON, T.B. & LANG, R.J. (1985). Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature,* **316,** 345–347.

BOLTON, T.B. (1976). On the latency and form of the membrane responses of smooth muscle to the iontophoretic application of acetylcholine or carbachol. *Proc. R. Soc. B.,* **194,** 99–119.

CHAHL, L.A. (1983). The properties of the clonidine withdrawal response of guinea-pig isolated ileum. *Br. J. Pharmacol.,* **85,** 457–462.

COLLIER, H.O.J., CUTHBERT, N.J. & FRANCIS, D.L. (1981). Clonidine dependence in the guinea-pig ileum. *Br. J. Pharmacol.,* **73,** 443–453.

COSTA, M., BROOKES, S.J.H., STEELE, P.A., GIBBINS, I., BURCHER, E. & KANDIAH, C.J. (1996). Neurochemical classification of myenteric neurones in the guinea-pig ileum. *Neuroscience,* **75,** 949–967.

CUTLER, D.J. & MASON, R. (1995). Effects of opioid agonists on Syrian hamster suprachiasmatic nuclei (SCN) neurones *in vitro*. *Br. J. Pharmacol.,* **116,** 93P.

DAVID, C., DAVIS, N., MASON, R. & WILSON, V.G. (1993). Evidence for functional dissociation of dependence and tolerance in guinea-pig isolated ileal segments following 20 hour exposure to morphine *in vitro*. *Br. J. Pharmacol.,* **110,** 1522–1526.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: applications to bioassay, radioligand assay and physiological dose response curves. *Am. J. Physiol.,* **235,** E97–E102.

EMONDS-ALT, X., DOUTREMEPUICH, J-D., HEAULME, M., NELIAT, G., SANTUCCI, V., STEINBERG, R., VILIAIN, P., BICHON, D., DUCOUX, J-P., PROIETTO, V., BROECK, D.V., SOURRIE, P., LE FUR, G. & BRELIERE, J-C. (1993). *In vitro* and *in vivo* biological activities of SR140333, a novel potent non-peptide tachykinin NK1 receptor antagonist. *Eur. J. Pharmacol.,* **250,** 403–413.

FURNESS, J.B. & COSTA, M. (1987). *The Enteric Nervous System*, Edinburgh: Churchill Livingstone.

GALLIGAN, J.J. (1993). Differential inhibition of cholinergic and noncholinergic neurogenic contractions by μ opioid and α_2-adrenergic agonist in guinea-pig ileum. *J. Pharmacol. Exp. Ther.,* **265,** 375–383.

GOMEZ, R.S., GOMEZ, M.V. & PRADO, M.A.M. (1996). Inhibition of Na^+/K^+ ATPase by ouabain opens calcium channels coupled by acetylcholine release in guinea-pig myenteric plexus. *J. Neurochem.,* **66,** 1440–1447.

HOLZER, P. & MAGGI, C.A. (1994). Synergistic role of muscarinic acetylcholine and tachykinin NK-2 receptors in intestinal peristalsis. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **349,** 194–201.

JOHNSON, S.M. & FLEMING, W.W. (1989). Mechanisms of cellular adaptive sensitivity changes: application to opioid tolerance and dependence. *Pharmacol. Rev.,* **41,** 435–488.

JOHNSON, S.M., WILLIAMS, J.T., COSTA, M. & FURNESS, J.B. (1987). Naloxone-induced depolarization and synaptic activation of myenteric neurones in morphine-dependent guinea-pig ileum. *Neuroscience,* **21,** 595–602.

KONG, J.-Q., LEEDHAM, J.A., TAYLOR, D.A. & FLEMING, W.W. (1997). Evidence that tolerance and dependence of guinea-pig myenteric neurons to opioids is a function of altered electrogenic sodium-potassium pumping. *J. Pharmacol. Exp. Ther.,* **280,** 593–599.

LEEDHAM, J.A., KONG, J.-Q., TAYLOR, D.A., JOHNSON, S.M. & FLEMING, W.W. (1992). Membrane potential in myenteric neurones associated with tolerance and dependence to morphine. *J. Pharmacol. Exp. Ther.,* **263,** 15–19.

LIM, S.P. & BOLTON, T.B. (1988). A calcium-dependent rather than a G-protein mechanism is involved in the inward current evoked by muscarinic receptor stimulation in dialysed single smooth muscle cells of small intestine. *Br. J. Pharmacol.,* **95,** 325–327.

MALLARD, N.J., HUDSON, A.L. & NUTT, D.J. (1992). Characterisation and autoradiographical localization of non-adrenoceptor idazoxan binding sites in the rat brain. *Br. J. Pharmacol.,* **106,** 1019–1027.

MALTA, E., RAPER, C. & TAWA, P.E. (1981). Pre- and postjunctional effects of clonidine and oxymetazoline-like compounds in guinea-pig ileal preparations. *Br. J. Pharmacol.,* **73,** 355–362.

MUNDEY, M.K., ALI, A., MASON, R. & WILSON, V.G. (1997). A comparison of the effect of morphine and naloxone on ventral tegmental area, locus coeruleus, and suprachiasmatic nucleus neuronal activity *in vitro*. *Br. J. Pharmacol.,* **122,** 93P.

SATOH, E. & NAKAZATO, Y. (1992). On the mechanism of ouabain-induced release of acetylcholine from synaptosomes. *J. Neurochem.,* **58,** 1038–1044.

SUPRENANT, A. & NORTH, R.A. (1988). Mechanism of synaptic inhibition by noradrenaline acting at α_2-adrenoceptors. *Proc. R. Soc., Lond. B.,* **234,** 85–114.

TSOU, K., WU, S-X., LU, Y.-A. & WAY, E.L. (1985). Block of the hyoscine-resistant opiate withdrawal contracture of ileum by a new substance P antagonist [D-Arg^1,D-Phe^5,D-Trp^{7,9}Leu^{11}] substance P. *Eur. J. Pharmacol.,* **110,** 155–156.

VIZI, E.S. (1972). Stimulation by inhibition of (Na^+/K^+-Mg^{2+})-activated ATPase, of acetylcholine release in cortical slices from rat brain. *J. Physiol.,* **226,** 95–117.

(Received January 26, 1998
Revised March 31, 1998
Accepted April 2, 1998)