Br. J. Pharmac. (1980), **68**, 47–55

KINETICS OF MORPHINE-SENSITIVE [^{3}H]-ACETYLCHOLINE RELEASE FROM THE GUINEA-PIG MYENTERIC PLEXUS

JAMES A. DOWN & JOHN C. SZERB

Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

1 Longitudinal muscle-myenteric plexus preparations from the guinea-pig ileum were superfused at a constant rate while isotonic contractions were monitored.

2 The preparations were superfused with [^{3}H]-choline while stimulated supramaximally at 0.1 Hz followed by washout in the presence of hemicholinium-3. The evoked release of the label due to a second 0.1 Hz stimulation in the absence of an anticholinesterase was measured.

3 Evoked efflux of the label was initially fast followed by a slower phase.

4 Morphine reduced the size of the pool and the rate of the initial fast efflux and the size of the pool but not the rate of the slow efflux evoked by supramaximal stimulation.

5 Submaximal stimulation reduced only the size of the pools from which the fast and slow efflux originated.

6 Naloxone reversed the depression of contractions and evoked release produced by morphine.

7 Results suggest that 0.1 Hz stimulation releases [^{3}H]-acetylcholine simultaneously from two pools. The fast release may originate from spontaneously firing units whose rate of discharge is depressed by morphine, while the slow release originates from neurones which do not fire spontaneously and whose threshold to field stimulation is increased by morphine.

Introduction

The myenteric plexus-longitudinal muscle of the guinea-pig ileum is one of the most extensively investigated *in vitro* models for the action of opiates and their antagonists (Kosterlitz & Waterfield, 1975). Opiate agonists reduce in a stereo-specific manner the spontaneous release of acetylcholine (ACh) as well as the release of ACh and twitches evoked by low frequency stimulation (Cox & Weinstock, 1966; De La Lande & Porter, 1967; Paton & Zar, 1968; Lees, Kosterlitz & Waterfield, 1973). This action of opiates is assumed to be the result of their combination with opiate receptors in this tissue which are similar to those found in the central nervous system (Pert & Snyder, 1973).

Two types of morphine-sensitive neuronal elements have been identified in the myenteric plexus: spontaneously active units recorded extracellularly, whose firing rate is depressed by morphine (Sato, Takayanagi & Takagi, 1973) and by enkephalins (North & Williams, 1976). The firing rate of these units is enhanced by ACh (Ehrenpreis, Sato, Takayanagi, Comaty & Takagi, 1976). The spontaneous firing and the depressant effect of morphine are maintained in Ca^{2+}-free high Mg^{2+} solutions in which synaptic transmission is absent (Dingledine & Goldstein, 1976), suggesting that morphine inhibits the activity of these units directly by raising their firing threshold. The other type of morphine-sensitive neurone, recorded intracellularly, does not fire spontaneously and is hyperpolarized by morphine (North & Tonini, 1977). This hyperpolarization which is accompanied by a decrease in input resistance, increases the firing threshold of these neurones to intracellularly applied depolarizing pulses and to focal stimulation. From these observations, North & Tonini (1977) concluded that in the myenteric plexus, opiates depress ACh release resulting from field stimulation by preventing some of the neurones from reaching their firing threshold.

Two methods have been used to assess the effect of opiates on ACh release in the myenteric plexus-longitudinal muscle preparation: measurement of twitches resulting from low frequency (0.1 Hz or less) field-stimulation (Paton, 1957; Cox & Weinstock, 1966; Paton & Zar, 1968; Ehrenpreis, Greenberg & Belman, 1973; Kosterlitz & Waterfield, 1975; Sawynok & Jhamandas, 1976) or the assay of endogenous ACh released in the presence of an anticholinesterase

0007-1188/80/010047-09 $01.00

(Paton, 1957; De La Lande & Porter, 1967; Paton & Zar, 1968; Paton, Vizi & Zar, 1971). Anticholinesterases cause a prolonged contracture of the preparation (Cox & Lomas, 1972). Therefore, it is impossible to measure simultaneously the effect of opiates on twitch height and on ACh release. Since under most conditions drugs that depress endogenous ACh release also reduce contractions, results from these two kinds of experiments are accepted to be equivalent. However, recently it has become evident that this interpretation is not always correct. For instance, hexamethonium reduced ACh release but not twitch height. This discrepancy was interpreted by Waterfield & Kosterlitz (1973) as due to the depressant effect of hexamethonium on the repetitive firing of ganglion cells in the plexus due to cholinesterase inhibition (North & Nishi, 1973).

In the experiments described here, the effect of morphine on the evoked release of labelled ACh from the myenteric plexus was measured in the absence of cholinesterase inhibition. This approach was based on previous observations that after labelling ACh stores with [^{3}H]-choline, electrical stimulation at low frequencies enhances the release of only [^{3}H]-ACh (Szerb, 1976; Wikberg, 1977). The advantages of measuring [^{3}H]-ACh release without inhibiting cholinesterase were the following: it allowed the simultaneous measurement of release and contractions; the stimulant effect of accumulated extracellular ACh on opiate-sensitive units (Ehrenpreis *et al.*, 1976) and on postganglionic neurones (North & Nishi, 1973) was avoided; the possible inhibitory effect of accumulated extracellular ACh on further ACh release (Sawynok & Jhamandas, 1977; Kilbinger, 1977) was probably reduced. Furthermore, by measuring the release of labelled instead of endogenous ACh, the effect of morphine on the kinetics of ACh release could be established.

Methods

Experimental

The myenteric plexus-longitudinal muscle preparations were separated from approximately 4 cm long segments of ilea of multi-coloured guinea-pigs according to the method of Paton & Zar (1968). The preparations were weighed (average weight 41 mg, s.d. 6 mg) and then mounted in perfusion baths made up of 45 mm long pieces of 2 ml pipettes (i.d. 4 mm). For mounting, two threads tied to the two ends of the preparation were clamped by inserting the bottom of the bath into a groove on a No. 6 rubber stopper through which a stainless steel electrode and an 18 gauge needle serving as inflow tubing had been passed. A third long piece of thread tied to the middle of the myenteric plexus-longitudinal muscle preparation was placed around the larger wheel of a jewelled double pulley located above the bath and 0.6 g weight was attached to the end of the thread. The preparation when mounted was therefore folded in two, in the shape of an inverted V in order to accommodate more tissue in a small bath. The outflow at the top of the bath was through another 18 gauge tube, next to which the other stimulating electrode was placed but whose tip extended 5 mm beyond the tip of the outflow tube. The distance between the two electrodes was 35 mm and the bath contained 0.5 ml fluid. The bath was perfused at a rate of 0.4 ml/min with Krebs solution by two peristaltic pumps, one to deliver and the other to remove the solution from the bath. Two such perfusion baths were set up side by side and after the tissue was mounted in them, they were immersed in a 37°C constant temperature bath.

Stimuli (0.1 Hz, 1 ms, square wave) were delivered from the S_1 channels of two Grass S8 stimulators, while the S_2 channels closed and opened relays to provide pulses of alternating polarity. Stimulation with alternating polarity was used to avoid possible polarization of the electrodes during prolonged stimulation. The mechanical activity of the preparation was measured isotonically by means of L.V.D. transducers (Sanborn). For this purpose the moving core of the transducer was suspended on the smaller wheel of the double pulley and was balanced against an identical weight.

After setting up the preparation, it was continuously superfused with Krebs solution containing 1 μM choline. After 30 min superfusion at rest the tissue was stimulated for 1 h at 47 V, during which time contractions slowly increased. Then by increasing the voltage stepwise from submaximal, the voltage required to obtain maximal contractions was established. This maximal voltage was found to be between 30 and 38 V in different preparations. Then the preparation was stimulated at 1.3 times this voltage (supramaximal stimulation) for a further 30 min in all experiments. During the last 15 min of this supramaximal stimulation, the perfusion fluid contained 4 μCi/ml [^{3}H]-choline while maintaining the total choline concentration at 1 μM. After the labelling stimulation was stopped, superfusion with Krebs solution containing 1 μM unlabelled choline and 10 μM hemicholinium-3 (HC-3) was started. This drug was included in the perfusion fluid after labelling to prevent the uptake of [^{3}H]-choline originating from released [^{3}H]-ACh.

Collection of samples was begun 55 min after the end of labelling when spontaneous efflux approached an exponential rate. Samples were collected according to the following schedule: first, five 4 min samples were collected at rest to obtain the spontaneous out-

flow of the label. Then the tissue was stimulated for 64 min at either the same supramaximal voltage as during labelling or at submaximal voltage which was 2/3 of the maximal voltage. During the first 24 min of stimulation, twelve 2 min samples were collected followed by ten 4 min samples. After the stimulation was turned off, a further eight 4 min samples were collected. Morphine (1 μM) was present in the perfusion fluid from the end of labelling till the end of the experiment. Half ml of the samples was counted and conversion of ct/min to d/min was carried out by the external channel ratio method. Radioactivity released was expressed as pmol g^{-1} wet tissue, based on the specific activity of the [^{3}H]-choline solution used for labelling.

Drugs used were: [^{3}H-methyl]choline chloride 10.1 Ci/mmol (Amersham-Searle); morphine sulphate (BDH); hemicholinium-3 (Aldrich); naloxone (Endo); choline chloride (Eastman). The composition of the Mg-free Krebs solution was (mM): NaCl 120, $CaCl_2$ 2.6, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 10. Krebs solution to be delivered to the bath was constantly bubbled with 95% O_2 and 5% CO_2.

Calculation of the parameters of release

Evoked release It has been shown previously that the radioactivity released at rest from the myenteric plexus which had been incubated with [^{3}H]-choline can be separated into three components in the presence of physostigmine: a fraction not precipitated by ammonium reineckate, [^{3}H]-choline and [^{3}H]-ACh (Szerb, 1976; Wikberg, 1977). The fraction not precipitated by ammonium reineckate is probably [^{3}H]-phosphorylcholine (Szerb, 1975). Low-frequency (0.1 Hz) stimulation increases only the release of [^{3}H]-ACh measured in the presence of physostigmine (Szerb, 1976; Wikberg, 1977). Therefore, it can be assumed that the difference between the total radioactivity of stimulated and unstimulated samples measured in the absence of cholinesterase inhibition equals the amount of [^{3}H]-choline derived from released [^{3}H]-ACh. Previous observations have shown that the spontaneous release of radioactivity declines in an exponential fashion after an initial fast washout (Wikberg, 1977) and this exponential decline in release can be calculated by interpolating the spontaneous release measured at the beginning and at the end of stimulation (Szerb, 1976). Therefore, spontaneous release was calculated in each experiment from the first five samples obtained before stimulation and the last five samples when the effect of stimulation had subsided. Such calculated spontaneous efflux curves are shown in Figures 1 and 2. Evoked release was obtained by subtracting the calculated spontaneous release from the total release in samples collected during stimulation.

Rate constant and initial pool size It became apparent early in the experiments that the evoked release did not proceed at a single exponential rate: at the beginning of stimulation the efflux of radioactivity declined faster than towards the end. It was assumed that the reason for the initial fast and later slow efflux was that [^{3}H]-ACh was released from two parallel and independent pools. A further assumption was that release from each pool followed first order kinetics, that is the rate of release was proportional to the amount of [^{3}H]-ACh, n_i ($i = 1, 2$), in the given pool:

$$\frac{dn_i}{dt} = -k_i n_i \quad (i = 1, 2) \qquad (1)$$

where k_i for $i = 1$ is the rate constant of the fast and for $i = 2$ of the slowly releasing pool. Integration of equation (1) gives the amount of [^{3}H]-ACh (n_i) in either pool at time t as:

$$n_i = n_{i0}\, e^{-k_i t} \quad (i = 1, 2) \qquad (2)$$

where n_{i0} ($i = 1, 2$) are the respective initial pool sizes. The observed evoked release relates to the total pool size, $n = n_1 + n_2$ and is presented as the amount, Δn, of [^{3}H]-ACh released per unit time interval Δt. Since Δt is small relative to the overall course of efflux during the experiment, the ratio $\Delta n/\Delta t$ is a reasonable approximation to the derivative dn/dt. By eqns. (1) and (2) each of the two components dn_1/dt and dn_2/dt gives the following:

$$\frac{dn_i}{dt} = -n_{i0} k_i e^{-k_i t} \quad (i = 1, 2) \qquad (3)$$

From eqn. (3)

$$\log(-dn_i/dt) = \log(n_{i0} k_i) - k_i t \quad (i = 1, 2) \qquad (4)$$

Therefore, plotting the logarithm of efflux from each pool per unit time against time will give a straight line with a zero intercept log ($n_{i0} k_i$) and a slope of $-k_i$.

However, the observed data are the sum of the effluxes from the two pools. To obtain the rate of efflux of the label from each pool, first the slow efflux was analysed according to eqn. (4) by least square fitting of the logarithm of the efflux per min against time during the last 32 min of stimulation. As shown in Figure 4, these points fitted a straight line, the slope of which was the rate constant of the slow release. To obtain the efflux from the fast pool, the initial values of the slow efflux were calculated and these were subtracted from the total observed evoked release. The least square fitting of the logarithm of these differences against time during the first 12 min

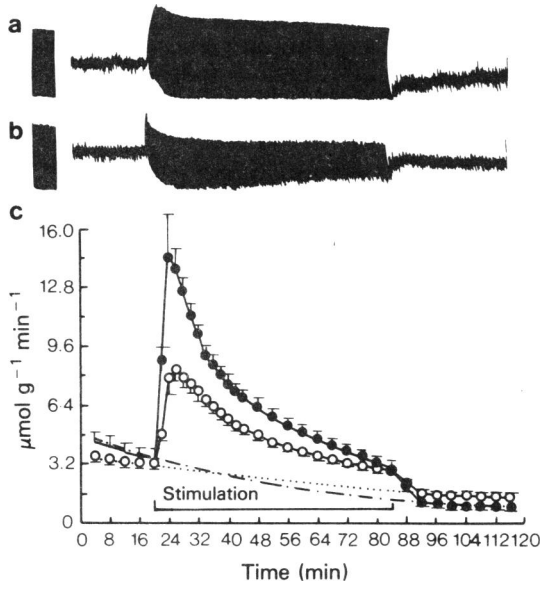

Figure 1 The effect of supramaximal stimulation on contractions and [^{3}H]-acetylcholine ([^{3}H]-ACh) release in the absence or presence of 1 μM morphine; (a) and (b) are examples of isotonic contractions recorded while measuring [^{3}H]-ACh release. Contractions shown at the beginning of the tracings are those caused by supramaximal stimulation during labelling. (a) Contractions in the absence of morphine, stimulation with 45 V during labelling and release; (b) contractions in the presence of 1μM morphine, stimulation with 47 V during labelling and release. Superfusion with morphine started after labelling; (c) release of the label (●——●) in the absence, (○——○) in the presence of morphine. Mean values of 6 experiments, bars denote s.e. mean; (—·—·) calculated spontaneous release in the absence and (····) in the presence of morphine (see Methods).

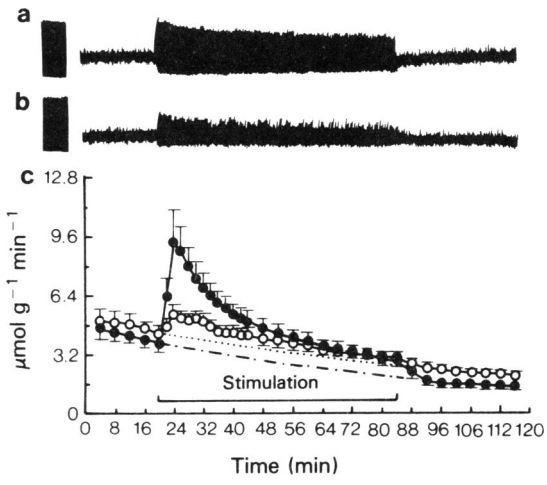

Figure 2 The effect of submaximal stimulation on contractions and [^{3}H]-acetylcholine ([^{3}H]-ACh) release in the absence or presence of 1 μM morphine; (a) and (b) examples of isotonic contractions recorded while measuring [^{3}H]-ACh release. (a) In the absence of morphine, stimulation with 45 V during labelling and 22 V during release; (b) in the presence of morphine after labelling, labelling with 47 V, releasing with 24 V; (c) release of label (●——●) in the absence or (○---○) presence of morphine. Mean values of 6 experiments, bars denote s.e. mean; (—·—·) calculated sponteneous release in the absence and (····) in the presence of morphine.

(six 2 min samples) after zero time gave the values for the fast efflux according to eqn. (4). As shown in Figure 4, these points followed a straight line, the slope of which gave the rate constant of the fast release.

The size of the individual initial pools (n_{i0}) was obtained from eqn. (3) which shows that the efflux at $t = 0$, $n_i(0)$, is:

$$\frac{dn_i(0)}{dt} = -n_{i0}k_i \quad (i = 1, 2) \qquad (5)$$

Thus, according to eqn. (5) the initial pool size (n_{i0}) is obtained in units of pmol g^{-1} by dividing the extra-

Table 1 Size of contractions and evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) during 64 min stimulation

Releasing stimulation	*Drug*	*Size of contractions** *At the start*	*At the end*	*Evoked release (pmol g^{-1})*	*% of release with supramax stimulation*
Supramaximal	—	117 ± 5	102 ± 4	276 ± 24	100
Supramaximal	Morphine 1 μM	75 ± 7	36 ± 5	151 ± 18	55
Submaximal	—	95 ± 3	60 ± 3	131 ± 31	47
Submaximal	Morphine 1 μM	37 ± 4	17 ± 1	36 ± 5	13

* % of contractions by supramaximal stimulation during labelling. Means ± s.e. mean of 6 observations.

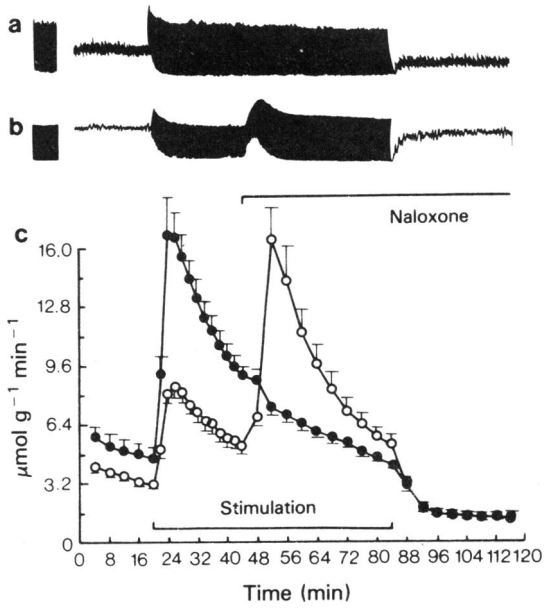

Figure 3 Effect of 0.5 μM naloxone on contractions and [^{3}H]-acetylcholine ([^{3}H]-ACh) release evoked by supramaximal stimulation in the absence or presence of 1 μM morphine. When naloxone was applied in the presence of morphine, superfusion with morphine was continued together with naloxone. (a) and (b) examples of isotonic contractions recorded while measuring [^{3}H]-ACh release. (a) Labelling and release with 42 V stimulation; (b) labelling and release with 47 V stimulation; (c), release of the label (●) in the absence or (○) presence of morphine. Mean values of 4 experiments; bars denote s.e. mean.

polated intercept, $n_{i0}k_i$, from eqn. (4), given in pmol g^{-1} min^{-1} by the rate constant, k_i, given in min^{-1}.

Because of the delay in the appearance in the collection vials of the label resulting from stimulation, zero time was chosen not to be the start of the stimulation but 4 min after it (Figure 4). Evoked release appearing in the vials between the start of stimulation and this zero time was apportioned to the fast and slowly releasing pools by extrapolating backward the calculated regression lines beyond zero time.

Results

The radioactivity in the samples collected before stimulation declined slowly with time (Figures 1 to 3). Stimulation caused a rapid increase in the efflux of radioactivity and this increase coincided in time with the twitches produced by the stimulation (Figures 1 to 3). At the beginning of the releasing stimulation without morphine being present, the preparation contracted more vigorously than during labelling: supramaximal stimulation caused significantly greater contractions than during labelling, while submaximal releasing stimulation was almost as effective initially as supramaximal stimulation during labelling (Table 1). However, the size of contractions declined during the 64 min releasing stimulation due to the presence of HC-3. This decline was much more marked with submaximal releasing stimulation or with morphine than with supramaximal stimulation (Table 1, Figures 1 and 2).

The release of [^{3}H]-ACh evoked by 64 min stimulation did not correspond completely with the size of contractions during release: in the absence of morphine, submaximal stimulation caused the release of less than half of the amount released by supramaximal stimulation yet contractions were reduced by less than half (Table 1). On the other hand, morphine reduced contractions caused by supramaximal stimulation somewhat more than the total evoked release (Table 1).

The depression by morphine of twitch size and evoked release was overcome by naloxone which, when applied during the last 40 min of supramaximal stimulation, caused a simultaneous increase in the tone and twitch size and a large efflux of the label. The same concentration of naloxone in the absence of morphine had no effect on either contractions or rate of release of the label (Figure 3). This indicated that the observed depressant effects of morphine were due to its action on opiate receptors.

Atropine (1 μM) caused a 90% reduction in contractions due to supramaximal stimulation but had no effect on the release of the label. On the other hand, perfusion with physostigmine (6.2 μM) in the absence of stimulation caused a transient increase in the efflux of the label and a prolonged contraction. This loss of the label was probably due to the stimulation of postganglionic cholinergic neurones and of the spontaneously firing units by accumulating ACh and prevented the identification of the released label as [^{3}H]-ACh under the same conditions as these experiments were carried out.

Kinetics of release of [^{3}H]-acetylcholine

The evoked release of the label declined during the 64 min stimulation, indicating that the pool from which this evoked release occurred was being depleted by the stimulation. Plotting the logarithm of the evoked release against time did not give a straight line because the initial efflux exceeded that expected from the release observed during the last 32 min of stimulation (Figure 4). This suggested that the evoked release originated from two pools of [^{3}H]-ACh, one releasing faster than the other. The rate constants of release from these two pools and the initial sizes of

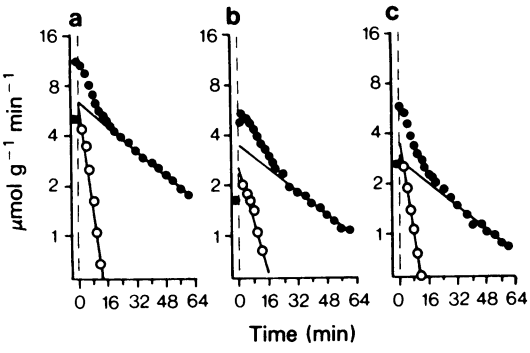

Figure 4 Semilogarithmic plots of the average evoked release of [^{3}H]-acetylcholine against time: (a) and (b) supramaximal releasing stimulation, (a) in the absence, (b) in the presence of 1 μM morphine; (c) submaximal releasing stimulation in the absence of morphine. (●) Total release, (○) calculated fast release. Lines show the calculated regression lines for slow and fast release. Time shown has been used for kinetic analysis (see Methods).

the pools in each experiment involving supramaximal stimulation or submaximal stimulation in the absence of morphine were obtained by the procedure described in the Methods. The evoked release with submaximal stimulation in the presence of morphine was too small and erratic to allow kinetic analysis.

As shown in Figure 4 and in Table 2, the rate constant of the late, slow release with supramaximal releasing stimulation was about 0.02 min^{-1} and was not reduced significantly either by morphine or when submaximal releasing stimulation was applied. However, morphine reduced the initial size of the slow pool to 55% of the control and this reduction was highly significant (Table 2). Submaximal releasing stimulation in the absence of morphine reduced the initial size of this pool slightly more, being only 44% of that observed with supramaximal stimulation.

The initial fast release with supramaximal stimulation originated from a pool whose size was only 1/6 of that of the slow pool but release from this pool occurred more than 8 times faster than from the slow pool with supramaximal releasing stimulation. In distinction to the slow pool, morphine slowed the rate of release from this fast pool by more than 50% and reduced the size of this pool slightly but significantly (Table 2). The size of the fast pool was also less with submaximal stimulation than with supramaximal stimulation, but the rate of release from this pool was not reduced significantly and was significantly higher than with supramaximal stimulation in the presence of morphine (Table 2).

Discussion

The somewhat larger contractions observed at the beginning of the releasing stimulation than during labelling, could have been due to the 75 min rest period between the two. During the 64 min releasing stimulation, the size of the contractions declined due to the presence of HC-3 which prevented the resynthesis of ACh released by stimulation (Szerb, 1976; Somogyi, Vizi & Knoll, 1977). However, the rate of decline was less with supramaximal stimulation in the absence of morphine than in its presence or with submaximal stimulation. Since supramaximal stimulation in the absence of morphine evoked the largest release of [^{3}H]-ACh, loss of ACh should have been more than in other conditions and therefore decline in contractions should also have been greater. The observation that the decline in twitch size was the least with supramaximal stimulation suggests that supramaximal stimulation releases more than adequate amounts of ACh from post-ganglionic nerve terminals hence provides a greater safety factor for contractions than submaximal stimulation or when morphine is present. Morphine appeared to decrease the size of contractions somewhat more than it depressed the evoked release of [^{3}H]-ACh with supramaximal stimulation

Table 2 Kinetic parameters of the evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh)

		Slow release		*Fast release*	
Releasing stimulation	*Drug*	*Initial pool (pmol g^{-1})*	*Rate constant (min^{-1})*	*Initial pool (pmol g^{-1})*	*Rate constant (min^{-1})*
Supramaximal	—	322 ± 31	0.0215 ± 0.0013	55.7 ± 5.3	0.1811 ± 0.0167
Supramaximal	Morphine 1 μM	182 ± 18**	0.0201 ± 0.0011	34.6 ± 7.2*	0.0862 ± 0.0072***‡
Submaximal	—	142 ± 20***	0.0197 ± 0.0010	34.9 ± 6.3*	0.1460 ± 0.0216

Means ± s.e. mean of 6 observations. Significance of differences (P) from supramaximal control: *$P < 0.01$; ***$P < 0.001$. ‡ Significantly different ($P < 0.05$) from submaximal control.

while submaximal stimulation in the absence of morphine produced larger contractions with less evoked release (Table 1). It is possible that morphine in addition to reducing the evoked release of ACh has a postjunctional non-specific depressant effect (Lewis, 1960).

The initial fast and the later slow rate of the exponential decline in evoked release suggests that stimulation releases [^{3}H]-ACh from two pools. The two pools are likely to release [^{3}H]-ACh in parallel because under the three conditions in which kinetic analysis of the efflux could be carried out, the estimated pool sizes and rate constants of the two pools varied independently from each other. The rate constant of release from the slowly releasing pool was the same with supra- or submaximal stimulation or in the presence of morphine. The reduction in release from the slow pool with the two latter conditions was due solely to a decrease in the size of the pool that could be released. Since in all experiments labelling of ACh stores was carried out under identical conditions (supramaximal stimulation in the absence of morphine) the portion of the slowly releasing pool which could not be released with submaximal stimulation or in the presence of morphine must have remained in the preparation. This portion of the pool, unavailable for release by stimulation, could have been the source of the larger spontaneous efflux of the label after the end of stimulation which was observed in these conditions (Figures 1 and 2). Naloxone reversed simultaneously the depression of twitch size and of release produced by morphine. It appears that naloxone caused this reversal by making available for release the part of the slowly releasing pool which could not be released when only morphine was present. This is suggested by the results in Figure 3 which show that the total evoked release with both morphine and naloxone present was about the same as in the absence of both, although naloxone was present only after most of the fast pool has been released.

The observation that only the size of the pool from which supramaximal stimulation evokes slow release but not the rate of this release was reduced by morphine, strongly supports the conclusions of North & Tonini (1977). They suggested that in the myenteric plexus the amount of ACh released by field stimulation is reduced in the presence of morphine because some of the postganglionic neurones are hyperpolarized to such an extent that they fail to produce an action potential, hence fail to release ACh. The majority of these neurones (type 1 of Nishi & North, 1973; S cells of Hirst, Holman & Spence, 1974) do not show spontaneous activity and usually fire only once with either orthodromic or antidromic focal stimulation (North & Tonini, 1977). Therefore an increase in the firing threshold by morphine in a population of neurones having different thresholds of firing will result in some not firing at all while others will continue to produce one action potential with every stimulus. Those which continue to fire in the presence of morphine will deplete their [^{3}H]-ACh store at the same rate as without morphine, while the [^{3}H]-ACh store of those neurones which cease to generate action potentials as a result of hyperpolarization will not be available for release. The observation that submaximal stimulation which also activates only a fraction of neurones, has the same effect on the kinetics of the slow evoked release as morphine, supports the above interpretation. The absence of any significant reduction in the rate of the slow release of [^{3}H]-ACh by morphine makes it unlikely that a reduction in the release from this pool is a result of any change in the excitation-release coupling such as a decrease in the depolarization-induced influx of Ca^{2+}. Such an effect would be expected to reduce primarily the rate of release but not the releasable pool size.

The initial fast release originates from a pool which is only about 1/6 of the slowly releasing pool but with supramaximal stimulation this release occurs more than 8 times faster than the slow release. Morphine reduced the rate of this fast release by more than 50% and decreased somewhat the size of the fast pool available for release. On the other hand, submaximal stimulation reduced only the available pool size but not the rate of the fast release. The small spontaneously-firing morphine-sensitive units (Sato *et al.*, 1973; Ehrenpreis *et al.*, 1976; Dingledine & Goldstein, 1976) which may be the terminal varicosities of post-ganglionic cholinergic neurones (North & Henderson, 1975) may be the origin of this fast releasing pool. If an action potential in these units results in the release of a given fraction of ACh present, a reduction in their rate of firing will result in a slower rate of release of [^{3}H]-ACh. Since the inhibitory effect of morphine in these units is probably the result of hyperpolarization (Dingledine & Goldstein, 1976) some of these units may fail to fire altogether in the presence of morphine and their [^{3}H]-ACh content will not be available for release, in the same way as submaximal stimulation may not activate all of these units. However, because these units fire spontaneously they may have lost some of their [^{3}H]-ACh content prior to the releasing stimulation. Therefore, the size of the fast releasing pool obtained here may have been underestimated by an unknown factor.

The labelling of ACh stores with a low concentration (1 μM) of [^{3}H]-choline in these experiments also underestimates the size of the ACh pools. It has been shown previously (Szerb, 1975) that the amount of [^{3}H]-ACh formed from [^{3}H]-choline increased about 10 times when the concentration of [^{3}H]-choline used for labelling ACh stores was increased from

1 to 50 μM. No further increase occurred with 250 μM [^{3}H]-choline. The smaller amount of [^{3}H]-ACh formed from low concentration of [^{3}H]-choline was ascribed to the dilution of [^{3}H]-choline by the large amount of endogenous unlabelled choline (Szerb, 1975). Thus, the true size of the two pools observed here is about 10 times larger than these results indicate, that is about 3.2 nmol g^{-1} for the slow and 0.55 nmol g^{-1} for the fast pool. If labelling of the slow pool proceeded at the same rate as release, during the 15 min exposure to [^{3}H]-choline, 27% of the pool should have been labelled. This would make the size of slowly releasing pool about 12 nmol g^{-1} or 12% of the approximately 100 nmol g^{-1} ACh present in the myenteric plexus (Szerb, 1975; Hutchinson, Kosterlitz & Gilbert, 1976). An additional pool of 7.5 nmol g^{-1} turns over only with high frequency stimulation (Szerb, 1976) while at least 55 nmol g^{-1} ACh turns over spontaneously even in the presence of tetrodotoxin or $MnCl_2$ (Szerb, 1975). These identified pools account for 75 nmol g^{-1} ACh. Thus the fast releasing pool is not likely to be larger than 25 nmol g^{-1}.

The observation that [^{3}H]-ACh is released from a fast and a slowly releasing pool is consistent with the observation on endogenous ACh release from this preparation. Initial pulses of trains of stimuli at frequencies higher than 0.1 Hz release more ACh and this initial large ACh release is more sensitive to the depressant effect of morphine and adrenaline than the smaller release evoked by the later pulses (Paton, 1963; Knoll & Vizi, 1971). This initial enhanced release may originate from the same pool as the fast release observed here. The ACh content of this fast releasing pool might not be replenished quickly enough to maintain a constant ACh output, especially when the availability of choline is limited due to cholinesterase inhibition. Therefore, subsequent pulses delivered in short intervals will release a higher proportion of ACh from the slowly releasing pool. Since morphine reduces both the rate of release and the size of the initial fast pool, but only the size of the slow pool, it will be more effective in inhibiting the larger release induced by the initial pulses of the train.

The authors wish to thank Dr I.W. Richardson for his advice and Mrs Joyce Armstrong and Mr Gary Gill for their excellent technical assistance. This work was supported by the Medical Research Council of Canada Grant DA-39.

References

Cox, B.M. & Lomas, D.M. (1972). The effects of eserine and neostigmine on the guinea-pig ileum and on ilial longitudinal muscle strips. *J. Pharm. Pharmac.*, **24**, 541–546.

Cox, B.M. & Weinstock, M. (1966). The effect of analgesic drugs on the release of acetylcholine from electrically stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **27**, 82–92.

De La Lande, I.S. & Porter, R.B. (1967). Factors influencing the action of morphine on acetylcholine release in the guinea-pig intestine. *Br. J. Pharmac. Chemother.*, **29**, 158–167.

Dingledine, R. & Goldstein, A. (1976). Effect of synaptic transmission blockade on morphine action in the guinea-pig myenteric plexus. *J. Pharmac. exp. Ther.*, **196**, 97–106.

Ehrenpreis, S., Greenberg, J. & Belman, S. (1973). Prostaglandins reverse inhibition of electrically-induced contractions of guinea-pig ileum by morphine, indomethacin and acetylsalicylic acid. *Nature, New Biol.*, **245**, 280–282.

Ehrenpreis, S., Sato, T., Takayanagi, I., Comaty, J.E. & Takagi, K. (1976). Mechanism of morphine block of electrical activity in ganglia of Auerbach's plexus. *Eur. J. Pharmac.*, **40**, 303–309.

Hirst, G.D.S., Holman, M.E. & Spence, I. (1974). Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *J. Physiol.*, **236**, 303–326.

Hutchinson, M., Kosterlitz, H.W. & Gilbert, J.C. (1976). Effects of physostigmine and electrical stimulation on the acetylcholine content of the guinea-pig ileum. *Eur. J. Pharmac.*, **39**, 221–235.

Kilbinger, H. (1977). Modulation by oxotremorine and atropine of acetylcholine release evoked by electrical stimulation of the myenteric plexus of the guinea-pig ileum. *Naunyn-Schmiedebergs Arch. Pharmac.*, **300**, 145–151.

Knoll, J. & Vizi, E.S. (1971). Effect of frequency of stimulation on inhibition by noradrenaline of the acetylcholine output from parasympathetic nerve terminals. *Br. J. Pharmac.*, **42**, 263–272.

Kosterlitz, H.W. & Waterfield, A.A. (1975). *In vitro* models in the study of structure activity relationships of narcotic analgesics. *A. Rev. Pharmac.* **15**, 29–48.

Lees, G.M., Kosterlitz, H.W. & Waterfield, A.A. (1973). Characteristics of Morphine-Sensitive Release of Neurotransmitter Substances. In *Agonist and Antagonist Actions of Narcotic Analgesic Drugs*. ed. Kosterlitz, H.W., Collier, H.O.J. & Villarreal, J.E. pp. 142–152. Baltimore: University Park Press.

Lewis, G.P. (1960). The inhibition by morphine of the action of smooth muscle stimulants in the guinea-pig intestine. *Br. J. Pharmac. Chemother.*, **15**, 425–431.

Nishi, S. & North, R.A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. *J. Physiol.*, **231**, 471–491.

North, R.A. & Henderson, G. (1975). Action of morphine on guinea-pig myenteric plexus and mouse vas deferens studied by intracellular recording. In *Opiate Narcotics, Neurochemical Mechanisms in Analgesia and Depen-*

dence. ed. Goldstein, A. pp. 217–220. New York: Pergamon Press.

NORTH, R.A. & NISHI, S. (1973). Properties of the ganglion cells in the myenteric plexus of the guinea-pig ileum determined by intracellular recording. In *Proceedings of the Fourth International Symposium on Gastro-Intestinal Motility*. ed. Daniel, E.E. pp. 667–676. Vancouver: Mitchell Press.

NORTH, R.A. & TONINI, M. (1977). The mechanism of action of narcotic analgesics in guinea-pig ileum. *Br. J. Pharmac.*, **61**, 541–549.

NORTH, R.A. & WILLIAMS, J.T. (1976). Enkephalin inhibits firing of myenteric neurones. *Nature, Lond.*, **264**, 460–461.

PATON, W.D.M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **12**, 119–127.

PATON, W.D.M. (1963). Cholinergic transmission and acetylcholine output. *Can. J. Biochem. Physiol.*, **41**, 2637–2653.

PATON, W.D.M., VIZI, E.S. & ZAR, A.M. (1971). The mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol.*, **215**, 819–848.

PATON, W.D.M. & ZAR, A.M. (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.*, **194**, 13–33.

PERT, C.B. & SNYDER, S.H. (1973). Opiate receptor: demonstration in nervous tissue. *Science, Washington*, **179**, 1011–1014.

SATO, T., TAKAYANAGI, I. & TAKAGI, K. (1973). Pharmacological properties of electrical activities obtained from neurons in Auerbach's plexus. *Jap. J. Pharmac.*, **23**, 665–671.

SAWYNOK, J. & JHAMANDAS, K.H. (1976). Inhibition of acetylcholine release from cholinergic nerves by adenosine, adenine nucleotides and morphine: antagonism by theophylline. *J. Pharmac. exp. Ther.*, **197**, 379–390.

SAWYNOK, J. & JHAMANDAS, K. (1977). Muscarinic feedback inhibition of acetylcholine release from myenteric plexus in the guinea-pig ileum and its status after chronic exposure to morphine. *Can. J. Physiol. Pharmac.*, **55**, 909–916.

SOMOGYI, G.T., VIZI, E.S. & KNOLL, J. (1977). Effect of hemicholinium-3 on the release and net synthesis of acetylcholine in Auerbach's plexus of guinea-pig ileum. *Neuroscience*, **2**, 791–796.

SZERB, J.C. (1975). Endogenous acetylcholine release and labelled acetylcholine formation from [^{3}H]-choline in the myenteric plexus of the guinea-pig ileum. *Can. J. Physiol. Pharmac.*, **53**, 566–574.

SZERB, J.C. (1976). Storage and release of labelled acetylcholine in the myenteric plexus of the guinea-pig ileum. *Can. J. Physiol. Pharmac.*, **54**, 12–22.

WATERFIELD, A.A. & KOSTERLITZ, H.W. (1973). Release of acetylcholine from the myenteric plexus of the guinea-pig ileum. In *Proceedings of the Fourth International Symposium on Gastro-Intestinal Motility*. ed. Daniel, E.E. pp. 659–666. Vancouver, Mitchell Press.

WIKBERG, J. (1977). Release of ^{3}H-acetylcholine from isolated guinea-pig ileum. A radiochemical method for studying the release of cholinergic neurotransmitter in the intestine. *Acta physiol. scand.*, **101**, 302–317.

(Received January 5, 1979.)