Effect of the putative cognitive enhancer, linopirdine (DuP 996), on quantal parameters of acetylcholine release at the frog neuromuscular junction

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- 1 The subcellular mechanism and site of action of linopirdine or DuP 996 (3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one) was investigated at the frog neuromuscular junction, using miniature endplate potential (m.e.p.p.) counts and a new method for obtaining unbiased estimates of n (number of functional release sites), p (probability of release), and var_sp (spatial variance in p).
- 2 DuP 996 produced an increase in m (no. of quanta released), which was due to an increase in n and p . The increase in m was concentration-dependent over a range of 0.1–100 μM and completely reversible with 15 min of wash. There was a saturation in the increase in p , but not in the increase in m and n , for [DuP 996] > 10 μM . By contrast, there was no major change in var_sp .
- 3 Block of presynaptic Na^+ - and Ca^{2+} -channels with 3 μM tetrodotoxin and 1.8 mM Co^{2+} prevented the m.e.p.p. frequency increase to DuP 996, and this effect was completely reversed by washing.
- 4 Application of the neuronal Ca^{2+} -channel blocker, ω -conotoxin GVIA (1 μM) brought about a rapid and profound decrease in the m.e.p.p. frequency increase produced by DuP 996. The effect of the toxin was not reversed by prolonged washing.
- 5 Block of voltage-gated K^+ -channels with 100 μM 4-aminopyridine (4-AP) resulted in only a small (28%) increase in m . The combination of 4-AP (100 μM) and DuP 996 (10 μM) produced an increase in m (189%) which was much greater than the sum of the responses to each agent alone. This increase in m was due solely to an increase in n , as p and var_sp were unchanged.
- 6 For [DuP 996] up to 100 μM , there was no apparent change in the mean size, amplitude distribution, or time course of m.e.p.ps, signifying that it had no anticholinesterase activity.
- 7 It is concluded that DuP 996 increases the release of quantal transmitter but not the postsynaptic response to the quanta. This appears to involve an effect at the nerve terminal membrane, most likely an increase in Ca^{2+} -conductance, and not an action to block K^+ -conductance or to release Ca^{2+} from intraterminal organelles.

Keywords: Acetylcholine; 4-aminopyridine; Ca^{2+} influx; ω -conotoxin GVIA; DuP 996; intracellular organelles; linopirdine; miniature endplate potentials; neurosecretion; quantal release parameters

Introduction

One of the widely held hypotheses on the pathophysiology underlying Alzheimer's disease (AD) is that of a cholinergic deficit in the nucleus basalis of Meynert (Whitehouse *et al.*, 1982; Katzman, 1986). Pharmacological approaches to providing a palliative treatment for AD have thus been aimed at enhancing transmission at the cholinergic synapses remaining in this region (Reinikainen *et al.*, 1990; Kumar & Calache, 1991). Agents affecting the cholinergic system which have been examined with varying success include arecoline (Christie, 1982), bethanechol (Penn *et al.*, 1988; Read *et al.*, 1990), physostigmine (Muramoto *et al.*, 1984; Harrell *et al.*, 1990), and tetrahydroaminoacridine (THA) (Summers *et al.*, 1986; Molloy *et al.*, 1991). Agents which have received more serious consideration are Synapton, a controlled release physostigmine (Forest Labs), Cognex, a formulation of THA (Warner-Lambert), Mentane, a THA analogue (Hoechst-Roussel), and HP 290, an analogue of physostigmine (Hoechst-Roussel) (reviewed by Sarter, 1991).

Linopirdine or DuP 996 (3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one, Du Pont Merck) is a compound currently undergoing Phase III testing for possible use in the treatment of AD. Preclinical studies have shown that DuP 996 enhances the K^+ -evoked release of acetylcholine (ACh), dopamine and 5-hydroxytryptamine (5-HT) in brain slices,

without affecting the resting release of these transmitters (Nickolson *et al.*, 1990). A similar increase in ACh release has been seen in synaptosomes (Vickroy, 1993). The ability to release ACh is highly correlated with the affinities of DuP 996 and thirty of its analogues for specific receptors (Tam *et al.*, 1991) located in areas of the brain associated with cognition (De Souza *et al.*, 1992). DuP 996 also enhances the performance of rats in behavioural tests and increases the amount of their alpha and beta wave activity recorded with EEGs (Cook *et al.*, 1990).

Recent studies with rodent skeletal muscle have shown that DuP 996 increases the direct and indirect twitch, the miniature endplate potential (m.e.p.p.) frequency, and the quantal content of the endplate potential (e.p.p.) in Mg^{2+} -depressed preparations (Tsai *et al.*, 1992). Despite this report, there remain several questions regarding the actions of DuP 996. First, it is possible that the release of ACh measured neurochemically consists of both quantal and non-quantal ACh. A significant increase in quantal ACh release should therefore be documented in order to relate the effect of DuP 996 on ACh output with that on cognitive function. Second, the increase in ACh output with DuP 996 may be due to either (a) an enhancement of the normal processes involved in neurosecretion or (b) an inhibition of, or damage to, critical cellular components, which could lead to leakage of ACh (e.g. DuP 996 could inhibit quantal packaging, in which case the increased output might be due to overflow of non-

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quantal ACh). Identification of the underlying mechanism is thus important to assess the ultimate effects of an agent earmarked for long-term patient use.

We have examined the effect of DuP 996 using a new method for obtaining the quantal release parameters, m (number of quanta released), n (number of functional release sites), p (probability of release), and var_p (spatial variance in p) (Provan & Miyamoto, 1993a). This approach circumvents the problem of nonuniformity and nonstationarity in the quantal estimates (Brown *et al.*, 1976), as well as the problem of inaccuracies in the nerve-evoked synaptic potential (Zucker, 1977). The aims of this study were (a) to examine whether DuP 996 produces a significant increase in quantal ACh release, as monitored by the release parameters, and (b) to elucidate the cellular or subcellular mechanism by which DuP 996 enhances neurosecretion. A preliminary account of these results was presented at the 1992 Meeting of the American Society for Pharmacology and Experimental Therapeutics in Orlando, FL (Provan & Miyamoto, 1992).

Methods

Experimental procedures

Conventional intracellular microelectrodes (3 M KCl, 8–15 M Ω) were used to record m.e.p.ps from isolated cutaneous pectoris muscles of frogs (*Rana pipiens*), after decapitating and double pithing the animals. Preparations were mounted in a 1 ml Perspex chamber which was continuously perfused with Ringer solution by means of a dual chambered pump. Junctions were selected on the basis of focal recording (m.e.p.p. rise time < 2 ms), large m.e.p.p. size (> 0.4 mV), moderately high m.e.p.p. frequency (> 10 Hz), good signal-to-noise ratio, few giant m.e.p.ps and sub-m.e.p.ps, and high and stable muscle resting potential. Continuous single junction recordings were held throughout each series of solution changes, and only one trial was carried out on each preparation. The problem of maintaining impalements for these periods was minimized by use of an air-suspension table and a hydraulic microdrive which allowed fine repositioning of the electrode. Tracings of resting potential, bath temperature, and experimental notations with time were made on a dual channel chart recorder. Studies were conducted at the ambient temperature (range 22–25°C), which varied less than 2°C during any single experiment.

Instrumentation

Bioelectric signals were fed into a high impedance preamplifier and viewed on an oscilloscope. Signal-to-noise ratio was increased by a band-pass filter set at 0–1 kHz. Signals were boosted by the rear-output amplifier of the oscilloscope to allow interfacing with an R.C. Electronics analogue-to-digital data acquisition unit (Goleta, CA). The unit was coupled to a Zenith Z-386SX computer with 2 MB of RAM and 40 MB hard drive, and data were analysed directly on-line or stored on FM magnetic tape for off-line analysis.

Experimental solutions and drugs

[K⁺] in the Ringer solution was elevated to 10 mM (equimolar substitution of KCl for NaCl) to raise the basal m.e.p.p. frequency and reduce the possibility of a Poisson-distributed release of transmitter (Provan & Miyamoto, 1993a). The control solution thus contained (in mM): NaCl 102.5, KCl 10.0, CaCl₂ 1.8, Tris 2.0 (adjusted to pH 7.2), and glucose 5.6. Muscles were equilibrated for at least 30 min before use, to allow re-establishment of the Donnan equilibrium and ensure that results were obtained during the long-term, steady-state effect of K⁺ (Cooke & Quastel, 1973). Tetrodotoxin (TTX)-Co²⁺ solution was made by adding 3 μ M TTX to the control solution and replacing the Ca²⁺ with

1.8 mM Co²⁺ to maintain a constant [divalent cation] and avoid changes in membrane surface charge (Hille, 1984). DuP 996 was dissolved in ethanol, with the final [ethanol] in the Ringer solution being 40 μ M. Although this was considered too low to affect transmitter release (Deitrich *et al.*, 1989), an equivalent amount of the vehicle was added to all solutions as a precaution. DuP 996 was obtained from The Du Pont Merck Pharmaceutical Co. (Wilmington, DE, U.S.A.), and TTX, CoCl₂ hexahydrate, 4-AP, and ω -conotoxin GVIA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Data collection, analysis and statistics

Specific details of the overall procedure are described elsewhere (Provan & Miyamoto, 1993a). Briefly, the number of quanta released by one nerve impulse (m) was replaced by the number of m.e.p.ps occurring in a 50 ms time interval (bin), and 500 sequential bins were used for each estimate. Data were divided into subgroups of 100 before analysis to minimize nonstationarity, and results which were nonstationary according to statistical tests were discarded. Unbiased estimates of m , n , p , and var_p were then computed by use of equations previously reported (Miyamoto, 1986).

M.e.p.p. amplitudes were obtained from digitized data and adjusted for baseline fluctuations with a double vertical cursor tool. Results were corrected for shifts in membrane potential (Katz & Thesleff, 1957), after recalculating the reversal potential for ionic changes in the solution (Takeuchi, 1963). The number required for each estimate was determined by collecting 500 m.e.p.ps and designating the mean amplitude as the 'control'. These were then divided into subgroups of 50, and the means of the cumulative subgroups plotted as percentages of control. A sample size of two subgroups (100 m.e.p.ps) was found to be sufficient, since accuracy was not improved with the use of more subgroups.

Results were obtained from 5–6 different preparations for each experimental procedure. In several instances, data were converted to percentage of control and then averaged, to illustrate better the percentage increase or decrease. In these cases, the values equal to 100% are given in the Figure legends. One-way analysis of variance (ANOVA) was used to test for differences between groups of data, with $P < 0.05$ chosen as the level of significance. Results are expressed as means $\pm$ s.e.mean.

Results

Effect of DuP 996 at K⁺-depolarized nerve terminals

The first step was to find a working concentration of DuP 996. Junctions were selected as described, and m.e.p.ps recorded every 5 min. DuP 996 was added to the bath starting at 0.1 μ M, and recordings made after 15 min. The concentration was then increased in half-logarithmic increments (0.3, 1, 3 μ M, etc.), and the response recorded after another 15 min. The time course of effect (onset, peak, and reversal) was found to be basically the same for each concentration tested, and the concentration finally used was 10 μ M. An illustration of the response is presented in Figure 1, in which sample records of m.e.p.ps are shown for control (Figure 1a), onset of effect (Figures 1b–d), and wash (Figure 1e).

The time course and magnitude of the response was examined in more detail by recording m.e.p.ps for 25 min in control solution, 60 min in 10 μ M DuP 996, and 25 min during wash. Data from six continuous single cell recordings were combined and plotted as m.e.p.p. frequency vs. time (Figure 2a). The results showed that, following a stable control period, addition of DuP 996 ($t = 0$, first dotted line) produced an immediate increase in m.e.p.p. frequency. The frequency reached a plateau after 15 min and remained at that level (179% of control) for the duration of exposure to

Figure 1 Effect of DuP 996 demonstrated by data from a representative experiment: m.e.p.ps were recorded continuously from a single junction, and sample traces (100 ms long for clarity) show m.e.p.ps: (a) in control 10 mM K^+ -Ringer (45.2 quanta s^{-1}); (b) 5 min after introducing 10 μ M DuP 996 (72.2 quanta s^{-1}); (c) 10 min with DuP 996 (73.8 quanta s^{-1}); (d) 15 min with DuP 996 (92.2 quanta s^{-1}); and (e) 15 min after washing out DuP 996 (40.6 quanta s^{-1}). Analogue signals were digitized with a 1 MHz R.C. Electronics data acquisition unit and printed with a Hewlett-Packard Laserjet III. The high signal-to-noise ratio is due to impalement of a small muscle fibre and low-pass filtering (1 kHz). Calibration bars: 1 mV (vertical) by 20 ms (horizontal).

DuP 996. Washing with control Ringer ($t = 60$ min, second dotted line) resulted in a decrease in m.e.p.p. frequency to control values within 15 min. The rapidity of recovery, as well as the similarity in time course of onset and washout, suggested that the effect of DuP 996 was readily reversible.

Effect of DuP 996 at K^+ -depolarized nerve terminals in the presence of TTX and Co^{2+}

The increase in m.e.p.p. frequency with DuP 996 (Figure 2a) could have been due to (a) an action at the nerve membrane to enhance Na^+ and/or Ca^{2+} influx, or (b) an action at an intracellular site which by-passed the membrane. One way of distinguishing between these two possibilities was to block the Na^+ - and Ca^{2+} -channels, then re-examine the response to DuP 996. Under these conditions, the absence of a m.e.p.p. frequency increase would imply that the effect of DuP 996 was mediated by Na^+ and/or Ca^{2+} influx, whereas a recurrence of the m.e.p.p. frequency increase would indicate that the site of action was further downstream.

The effect of Na^+ - and Ca^{2+} -channel block (using 3 μ M TTX and 1.8 mM Co^{2+} , respectively) on the response to DuP 996 was examined by recording m.e.p.ps for 15 min in control solution, 10 min in TTX- Co^{2+} solution, 60 min in TTX- Co^{2+} solution plus 10 μ M DuP 996 and 25 min during wash. Data from six trials were combined to give Figure 2b.

Figure 2 (a) Effect of DuP 996 on m.e.p.p. frequency (F): m.e.p.ps were continuously recorded from single junctions exposed to 10 mM K^+ -Ringer (control), 10 μ M DuP 996, and during wash. Data were normalised to percentage of the control values at time (t) zero. Addition of DuP 996 ($t = 0$) caused a rapid, sustained increase in m.e.p.p. F, and washing with 10 mM K^+ -Ringer ($t = 60$ min) produced a prompt, complete reversal of effect (100% = 46.5 m.e.p.ps s^{-1}). (b) Effect of tetrodotoxin (TTX) and Co^{2+} on the m.e.p.p. F increase produced by DuP 996. Experiments were identical to those above except that TTX- Co^{2+} Ringer was added 10 min prior to DuP 996. In the continued presence of TTX and Co^{2+} , the m.e.p.p. F increase expected with DuP 996 was completely blocked. M.e.p.p. F returned quickly to control levels after washing with control Ringer (100% = 37.7 m.e.p.ps s^{-1}). Points in (a) and (b) represent means $\pm$ s.e. means of six experiments each.

As anticipated, there was a marked decrease in the control m.e.p.p. frequency on exposure to TTX- Co^{2+} solution ($t = -10$ min in Figure 2b). In the continued presence of TTX and Co^{2+} , addition of 10 μ M DuP 996 ($t = 0$ min, first dotted line) failed to produce either an immediate or delayed increase in m.e.p.p. frequency (monitored for 60 min exposure to DuP 996). The inhibitory effect of TTX and Co^{2+} was completely reversed on return to control solution ($t = 60$ min, second dotted line), except for a slight overshoot and return to baseline at $t = 80$ min (this may have been a rebound effect due to the prolonged block or a delayed increase due to residual amounts of DuP 996).

Time course of effect of DuP 996 on unbiased quantal release parameters

The increase in m.e.p.p. frequency in K^+ -depolarized preparations (Figure 2a) was, according to the method of Provan & Miyamoto (1993a), equivalent to an increase in m . This increase in m could in turn be due to an increase in n , p , or both n and p (since $m = np$). To dissociate the effect on m into its quantal components and examine the temporal relationships of the changes, data were recorded for 15 min in control solution, 60 min in 10 μ M DuP 996 and 15 min of wash. Results from six experiments were combined and the effect on each parameter plotted as a function of time (Figure 3).

Figure 3 Effect of $10\ \mu\text{M}$ DuP 996 on unbiased estimates of quantal release parameters as a function of time. Continuous single cell recording was maintained throughout for control ($t = -15$ min), DuP 996 ($t = 0$ min), and wash ($t = 60$ min). Plots show effect of DuP 996 on (a) number of quanta released, m ; (b) number of functional release sites, n ; (c) probability of release, p ; and (d) spatial variance in p , $\text{var}_s p$. Note the increase in m , n and p but not $\text{var}_s p$. Values for m and n are in quanta, whereas p and $\text{var}_s p$ are dimensionless. Points represent means $\pm$ s.e.mean of six separate experiments.

As before (Figure 2a), the effect of DuP 996 on m occurred rapidly, with a peak increase after 10 min and a plateau thereafter (Figure 3a). There were also immediate increases in n and p (Figure 3b, c), which meant that the increase in m was due to increases in both parameters. By the end of 60 min exposure to DuP 996, there was a slight decrease in n (Figure 3b). This was offset by a small increase in p (Figure 3c), so the net effect was a sustained increase in m (Figure 3a) (the decrease in n may have been due to a reduced number of available quanta during the prolonged release of transmitter). On washing with control solution, the increases in n and p , like that for m , returned to control levels within 15 min (Figures 3a–c). By contrast, DuP 996 produced no significant changes in $\text{var}_s p$ (Figure 3d).

Concentration-dependent effects of DuP 996 on unbiased quantal release parameters

The effect of [DuP 996] on m , n , p and $\text{var}_s p$ was examined to provide information on (a) the threshold concentration which elicited a response, (b) the nature of the concentration-effect relationship, and (c) whether desensitization or inhibition occurred at the higher levels.

Junctions were sequentially exposed to half-logarithmic increments of [DuP 996] from 0.1 to $100\ \mu\text{M}$, and m.e.p.s recorded after 15 min exposure to each concentration (as the

response took 10–15 min to reach plateau). The combined results from six experiments showed concentration-dependent increases in m , n and p (Figures 4a–c), with the threshold effect occurring at $0.3\ \mu\text{M}$ (Figure 4a). The effect on p appeared to peak at [DuP 996] $> 10\ \mu\text{M}$ (Figure 4c), such that the increase in m at this level was due primarily to an increase in n (Figure 4b). The 50% effective concentration (EC_{50}) for p (using 4-parameter logistic curve-fitting) was $1.1\ \mu\text{M}$ (equivalent EC_{50} could not be determined accurately for m and n since these curves did not saturate). Lastly, increasing [DuP 996] produced no significant effects on $\text{var}_s p$ (Figure 4d), except for a modest increase at the highest concentration examined ($100\ \mu\text{M}$).

Effect of ω -conotoxin GVIA (ω -CgTX) on the increase in quantal release produced by DuP 996

The effect of ω -CgTX, an irreversible N-type Ca^{2+} -channel blocker (Sher & Clementi, 1991), was tested to elucidate further the mechanism of action of DuP 996. Recordings were carried out for 20 min in control solution, 30 min in DuP 996 ($10\ \mu\text{M}$), 7 min in DuP 996 plus ω -CgTX ($1\ \mu\text{M}$), and 23 min of wash (Figure 5). As before, DuP 996 produced a rapid increase in m.e.p.p. frequency (Figure 5, first dotted line) which was maintained at 165% of control. Subsequent

Figure 4 Concentration-effect curves of [DuP 996] vs. unbiased estimates of quantal release parameters, (a) number of quanta released, m , (b) number of functional release sites, n , (c) probability of release, p , and (d) spatial variance in p , $\text{var}_s p$. Data were continuously recorded from six junctions exposed first to control $10\ \text{mM}$ K^+ -Ringer, then to increasing [DuP 996]. Results (except those for $\text{var}_s p$) are normalised to percentages of the values obtained in the absence of DuP 996 (points represent means $\pm$ s.e.mean). Results show a marked concentration-dependent increase for each of the parameters except $\text{var}_s p$, with no indication of inhibition (reversal in effect) at the highest concentrations. Note the logarithmic scale for the abscissae. 100% = 1.09 quanta for m , 6.70 quanta for n , and 0.165 for p .

Figure 5 Effect of ω -conotoxin-GVIA (ω -CgTX) on the m.e.p.p. frequency (F) increase produced by DuP 996: m.e.p.ps were continuously recorded from junctions exposed to 10 mM K^+ -Ringer control solution, 10 μ M DuP 996, DuP 996 plus 1 μ M ω -CgTX, and during wash. Experiments were identical to those in Figure 2a, except for addition of ω -CgTX during the exposure to DuP 996. An aliquot of stock ω -CgTX solution was added to the chamber (to give a final concentration of 1 μ M) and perfusion stopped for 7 min (at $t = 31$ min). Data were normalised to percentages of the values at time zero. The inhibition produced by the toxin was not reversed by washing with either control solution (three trials) or control solution with DuP 996 (three trials), so these data were simply combined. Points represent means $\pm$ s.e.mean for six experiments (100% = 40.2 m.e.p.ps s^{-1}).

addition of 1 μ M ω -CgTX (second dotted line) produced a rapid and dramatic decline in m.e.p.p. frequency, which continued to near zero and which was not reversed with > 20 min of wash. These results indicated that the effect of DuP 996 was mediated primarily by voltage-gated N-type Ca^{2+} -channels in the presynaptic membrane.

Effect of DuP 996 in the presence of 4-aminopyridine (4-AP)

Influx of Ca^{2+} through voltage-gated Ca^{2+} -channels could be the result of either an increase in Ca^{2+} -conductance or a decrease in K^+ -conductance (which would indirectly increase Ca^{2+} -conductance by depolarizing the membrane (Harvey, 1993)). One way of differentiating between these two mechanisms was to examine the effect of DuP 996 after voltage-gated K^+ -channels were completely blocked, e.g., with supramaximal concentrations of 4-AP (Lundh, 1978). An increase in transmitter release under these circumstances would then indicate that the effect of DuP 996 could not have been due to block of voltage-gated K^+ -channels.

Figure 6 Concentration-effect curve of 4-aminopyridine (4-AP) vs. m.e.p.p. frequency (F). Data were continuously recorded from junctions exposed to 10 mM K^+ -Ringer solution (control), then to increasing [4-AP]. Results are normalized to percentages of values obtained in the absence of 4-AP. Note the logarithmic scale for the abscissae. Points represent means $\pm$ s.e.mean for five trials (100% = 30.5 m.e.p.ps s^{-1}).

Preliminary experiments were carried out to re-examine the concentration-dependent effects of 4-AP on m.e.p.p. frequency. Junctions were exposed to increasing [4-AP] in half-logarithmic increments up to 300 μ M, and m.e.p.ps recorded after 20 min perfusion at each concentration. The results (Figure 6) revealed a biphasic effect, with m.e.p.p. frequency decreasing slightly for [4-AP] < 30 μ M (cf. Molgó *et al.*, 1977) and increasing slightly for concentrations > 30 μ M. A concentration of 100 μ M 4-AP was chosen since it produced a measurable effect on m.e.p.p. frequency (Bowman *et al.*, 1977 and Figure 6) and on synaptic delay (Molgó *et al.*, 1977).

To examine the effect of voltage-gated K^+ -channel block on the response to DuP 996, recordings were carried out for 20 min in control solution, 50 min in 100 μ M 4-AP, 30 min in 100 μ M 4-AP plus 10 μ M DuP 996, and 25 min during wash (Figure 7). As before (Figure 6), 4-AP produced only a modest increase in m (to 128% of control). However, in the continued presence of 4-AP, addition of DuP 996 produced a marked increase in m to 289% of control. This was 117% greater than the average increase of 72% produced by DuP 996 alone (Figures 2a, 5). The increase in m was due to an increase in n (Figure 7b), as both p and var_p were unchanged (Figures 7c, d). The effect was reversed on washing, with a return to control levels in 15–20 min. The marked response in the presence of 4-AP indicated that

Figure 7 Effect of 4-aminopyridine (4-AP) on the response to DuP 996 with regard to (a) number of quanta released, m , (b) number of functional release sites, n , (c) probability of release, p , and (d) spatial variance in p , var_p . M.e.p.ps were continuously recorded from single junctions exposed to 10 mM K^+ -Ringer, 100 μ M 4-AP, 4-AP plus 10 μ M DuP 996, and wash. Addition of 4-AP ($t = 0$) produced only a modest increase (28%) in m . The response to DuP 996 ($t = 50$ min) was not attenuated but enhanced (by 189%) by the presence of 4-AP, and washing with control Ringer ($t = 81$ min) produced a complete reversal of effect. Note that the increase in m was due solely to an increase in n , as p and var_p were unaffected. Points represent means $\pm$ s.e.mean of six experiments.

DuP 996 was not working by decreasing voltage-gated K^+ -conductance.

Effect of DuP 996 on postsynaptic sensitivity

The effect of DuP 996 on m.e.p.p. amplitude was examined to test for alterations in postsynaptic sensitivity; e.g., an increase in m.e.p.p. size might indicate a drug action to (a) inhibit cholinesterase (ChE), (b) increase receptor sensitivity to ACh, or (c) potentiate ion fluxes resulting from postsynaptic receptor activation.

Digitized data from the concentration-effect experiments (Figure 4) were retrieved from storage. Amplitudes of 100 m.e.p.ps were measured and corrected for differences in muscle resting potential, for the control period and for each sequential increase in [DuP 996] from 0.1 to 100 μM (recalling that results were recorded only during the plateau phase occurring 15 min after changing solutions). The results showed that DuP 996 produced no significant changes in m.e.p.p. amplitude at any of the concentrations tested ($P > 0.05$, ANOVA).

Discussion

Unbiased estimates of quantal release parameters

The present study uses a new procedure which avoids the problem of nonuniformity and nonstationarity (Brown *et al.*, 1976) to obtain unbiased estimates of n and p (Provan & Miyamoto, 1993a). Important features of the method are the use of direct quantal counts (m.e.p.ps) and elevated K^+ to evoke transmitter release. Direct counting of quanta eliminates the need to measure e.p.p. amplitudes, which may give inaccurate estimates of m (Zucker, 1977) and which may reflect, at least in theory, both quantal and non-quantal release (Zemková *et al.*, 1990). Use of elevated K^+ mimics the conditions used in measuring total ACh release, thereby allowing a more direct comparison between results obtained for quantal and total ACh release.

Comparison of results with neurochemical studies

The present findings are in basic agreement with those of neurochemical studies which monitored K^+ -stimulated ACh release in brain slices (Nickolson *et al.*, 1990) and synaptosomes (Vickroy, 1993). For example, DuP 996 was found to produce a sizeable increase in quantal ACh release (Figures 1, 2a, 3–5), which was consistent with the increase in total (quantal plus non-quantal) ACh release found by Nickolson *et al.* (1990). Second, the threshold concentration for the effect on m was 0.3 μM (Figure 4a), which compared favourably with the 0.1 to 1 μM range found for brain slices (Table 1 of Nickolson *et al.*, 1990) and synaptosomes (Figures 2–4 of Vickroy, 1993). Third, the increase in m (to 300% of control with 100 μM , Figure 4a) was comparable to the average increase in ACh release for cortex, hippocampus, and caudate nucleus (to 335% of control with 100 μM ; Table 1 of Nickolson *et al.*, 1990) and for hippocampal synaptosomes (to 260% of control with 30 μM ; Figures 3, 4 of Vickroy, 1993). Finally, the effect of DuP 996 on m , n and p was readily reversible (Figures 2a, 3a, 7), which was consistent with the notion of a saturable, reversible binding to a specific receptor site (Tam *et al.*, 1991). While a direct comparison obviously cannot be made, the similarity of results between quantal and total ACh release does suggest that quantal release may comprise a substantial fraction of the total ACh release evoked by DuP 996.

DuP 996 acts at the presynaptic membrane

Several lines of evidence suggest that DuP 996 promotes ACh release by an action at the nerve terminal membrane. First, the effect of the drug is readily reversible (Figures 2a, 3a, 7), and the time course of onset is similar to that for washout. Both these findings are compatible with an extracellular rather than an intracellular site of action. Second, the effect of DuP 996 is reversibly inhibited by TTX and Co^{2+} (Figure 2b), which suggests that the action must occur at, or prior to, influx of Ca^{2+} at the membrane. Third, the effect of DuP 996 is rapidly and irreversibly blocked by ω -CgTX (Figure 5). This indicates the site of action may be at, or near, the voltage-gated Ca^{2+} -channel. Fourth, DuP 996 does not produce an increase in var_p (Figures 3d, 4d, 7d). According to recent studies (Provan & Miyamoto, 1993a), this indicates that DuP 996 does not work by releasing Ca^{2+} from intracellular organelles (see below). Taken together, the results are consistent with a drug action at an extracellular site on the presynaptic membrane.

Alterations in spatial variance in p

The use of var_p to distinguish between a drug action at intracellular organelles vs. one at the membrane is based on the premise that release of Ca^{2+} from intracellular organelles produces a non-uniform increase in $[Ca^{2+}]_i$ at transmitter release sites, which results in an increase in var_p . By contrast, influx of Ca^{2+} through Ca^{2+} -channels adjacent to transmitter release sites is presumed to produce a uniform increase in $[Ca^{2+}]_i$, which would produce no increase in var_p (Figure 8 of Provan & Miyamoto, 1993a). Accordingly, the absence of effect on var_p (Figures 3d, 4d, 7d) would be consistent with the idea that DuP 996 does not promote Ca^{2+} -release from internal organelles. An increase in var_p did occur at very high levels (100 μM), which suggests that DuP 996 may eventually enter the terminal and release Ca^{2+} from intracellular organelles.

Effects of DuP 996 on quantal release parameters resemble effects associated with Ca^{2+} influx at the terminal

The concentration-dependent effects of DuP 996 on quantal transmitter release include an increase in m , n and p (Figures 4a, b, c) a saturation in p at the higher concentrations, and no change in var_p (Figure 4d). This pattern of response is the same as that found with elevating $[K^+]_o$ or $[Ca^{2+}]_o$ (Provan & Miyamoto, 1993a) (the link being that both manoeuvres enhance influx of Ca^{2+} at the terminal). The similarity in the response to DuP 996 further suggests that DuP 996 acts, like elevated K^+ or Ca^{2+} , to promote Ca^{2+} influx at the presynaptic membrane.

Possible mechanism of action of DuP 996

DuP 996 acts in a selective manner, since it enhances the release of ACh, dopamine and 5-HT but not noradrenaline (NA) or γ -aminobutyric acid (GABA). However, it is not associated with activation of nicotinic or muscarinic receptors (Nickolson *et al.*, 1990), and it does not involve histamine, benzodiazepines, opioids, or a variety of peptides (Tam *et al.*, 1991).

DuP 996 does not act by inhibiting voltage-gated K^+ -conductance, because (a) block with 4-AP produces only a small increase in m.e.p.p. frequency (Figures 6, 7a), and (b) the effect of DuP 996 is not prevented by prior block with 4-AP (Figure 7a). These findings coincide with the report that DuP 996 does not inhibit voltage-gated K^+ -channels (Nickolson *et al.*, 1990). A block of calcium-activated K^+ -channels by DuP 996 is unlikely, because block of these channels with charybdotoxin produces no increase in m.e.p.p. frequency (Robitaille & Charlton, 1992). Finally, block of

K⁺-channels would have been manifested by a prolongation of the nerve action potential, which is not observed with DuP 996 (Tsai *et al.*, 1992).

This means that DuP 996 works by depolarizing the presynaptic membrane or by increasing Ca²⁺-channel conductance. While we cannot at present differentiate between these two possibilities, we tend to favour the latter explanation. The results with ω -CgTX (Figure 5) suggest that DuP 996 promotes the influx of Ca²⁺ through N-type Ca²⁺-channels. If this is so, it may be that the action is not specific for a particular type of Ca²⁺-channel, since ACh release at mammalian junctions, which is not mediated by N-type-Ca²⁺-channels (Wray & Porter, 1993), is also enhanced by DuP 996 (Tsai *et al.*, 1992).

The increase in *m* which results from 4-AP plus DuP 996 (Figure 7a) is of interest for two reasons: first, the increase is due solely to an increase in *n* (Figure 7), which is what is found with tetraethylammonium (Volle & Branisteanu, 1976) and guanidine derivatives (Volle & Branisteanu, 1976; Farley *et al.*, 1979) using nerve-evoked e.p.ps and biased estimates of *n* and *p*. Second, assuming that DuP 996 increases Ca²⁺-channel conductance, the finding of a supra-additive response with 4-AP would be compatible with the idea of overlapping effects of Ca²⁺ and K⁺ channels at the active zone (cf. Robitaille *et al.*, 1993).

DuP 996 does not alter postsynaptic sensitivity

DuP 996 produced no significant change in m.e.p.p. amplitude, and no visible change in m.e.p.p. rise time or decay (e.g., Figure 1). Accordingly, DuP 996 must have produced little or no ChE inhibition, which is in agreement with the neurochemical findings (Nickolson *et al.*, 1990). This lack of effect differs from the increases in m.e.p.p. amplitude found with 3 μ M THA (22%), physostigmine (46.5%) and neostig-

mine (79.2%) (Provan & Miyamoto, 1991), all of which are known antiChEs. This again implies that the facilitatory action of DuP 996 on cholinergic transmission must be due to pre- and not postsynaptic effects.

Potential advantages of DuP 996 as a palliative treatment for AD

One way of enhancing cholinergic transmission in AD is to inhibit ChE. However, chronic administration of antiChEs may produce receptor desensitization, so an alternative is to increase ACh release at the remaining nerve terminals, using agents such as DuP 996. The finding of an increase in *quantal* ACh release with DuP 996 affirms that the increase in ACh output is not due to interference with quantal packaging and overflow of non-quantal ACh, as may occur with physostigmine (Alderdice, 1979; Provan & Miyamoto, 1991).

An agent used in the treatment of AD should work by accelerating existing processes and not by inhibiting cellular components. Agents known to inhibit mitochondrial function, e.g., La³⁺, Hg²⁺, methyl Hg⁺, and flufenamic acid, have been found to increase *p* and var_p (Provan & Miyamoto, 1993a,b). Accordingly, the finding that DuP 996 does not alter var_p is important, since it implies that facilitation of ACh release is probably not due to mitochondrial inhibition. The absence of such adverse effects would be advantageous for DuP 996 or any other agent relegated to long-term clinical use.

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