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Attenuation by *N*-ethylmaleimide treatment of the cholinergically induced shortening of action potential duration in guinea pig right atrium

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Parasympathetic stimulation of the heart evokes, among other responses, a rapid and profound bradycardia [1]. Such a response now appears to be mediated, in part, by a cholinergically induced increase in K^+ efflux specifically from the atrial myocardium [2]. An initial clue as to the underlying cellular mechanism was the observation that this hormone-stimulated efflux could be mimicked by the intracellular application of GTP* analogues [3, 4]. These findings thus implicated a role for a guanine nucleotide binding (G) protein in the hormone signalling process, analogous to the involvement of stimulatory and inhibitory G-proteins in the hormonal mediation of adenylyl cyclase [ATP pyrophosphate-lyase (cycling), EC 4.6.1.1]. In support of this hypothesis, it was reported that pertussis toxin blocks the cholinergic activation of this atrial K^+ efflux [4–6]. Furthermore, re-addition of an activated G_i-like protein to such a blocked pathway restores hormonal sensitivity [7]. Despite these conceptual advances, the exact nature of this channel-associated heteromeric G_i-like protein or the active subunit constituent still remains controversial.

Biochemical characterization of the G-protein linked signalling pathway mediating inhibition of cardiac adenylyl cyclase reveals that the integrity of this mechanism is disrupted by alkylation of sulfhydryl (-SH) groups associated with one or more of the pathway components [8]. Decreases in both agonist binding and the degree of cholinergic inhibition have been noted. More specifically, NEM blocks the pertussis toxin catalyzed ADP-ribosylation of G_i-like proteins [9, 10].

In this study, we used NEM as a probe to examine whether the cholinergic signalling mechanism mediating shortening of action potential (AP) duration in mammalian cardiac right atrium involved critical -SH groups. The results indicate that treatment with NEM interfered with cholinergic attenuation of AP duration, through what appears to be disruption the G-protein(s)/cholinergic receptor interaction, as judged by changes in agonist binding characteristics.

Methods

Guinea pigs (male, 300–400 g) were stunned by a sharp blow to the head, followed by cervical dislocation. The hearts were quickly removed and placed in oxygenated (95%/5% O_2/CO_2) HEPES-buffered Tyrode's solution of the following composition (in mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 11.9 $NaHCO_3$, 5 HEPES, 11 dextrose; pH adjusted to 7.3–7.4 with 1 M HCl. Right atria were dissected from the heart and longitudinal strips of muscle (trabeculae) were isolated. Atrial strips were pinned to the Sylgard resin base of a recording chamber (volume = 3 ml) and allowed to equilibrate at least 30 min under low frequency (≈1 Hz) stimulation. Chamber perfusion was ≈1 ml/min.

Preparations were stimulated (Grass S88 stimulator) at one end, using bipolar electrodes, by square wave pulses (1.5–2.5 msec, 1.5–3 V, 2–3 Hz). Action potentials were recorded by a single intracellular microelectrode (tip resistance = 15–30 MΩ) filled with 3 M KCl; a Ag:AgCl agar bridge was used as the indifferent electrode. The recording electrode was connected to a Dagan 8500 preamplifier, and the action potentials and their first derivatives were displayed on a Tektronic 5111 storage oscilloscope and then photographed.

Competitive radioligand binding studies using rabbit atrial homogenates were carried out as previously described [11]. Intact atria (0.2–0.3 g) were incubated for 25 min in oxygenated Tyrodes' solution containing 100 μM NEM and then homogenized in cold 10 mM $NaKPO_4$ buffer at pH 7.4 (10%, w/v) using a PT-10 Polytron. Assay incubations were carried out for 1 hr at 25° in a 1-ml volume containing 10 mM $NaKPO_4$ at pH 7.4, ≈175 pM [^{3}H](−)QNB, competing drugs, guanine nucleotides, and 150–200 μg of frozen homogenate protein. Non-specific binding was determined in the presence of 1 μM atropine sulfate. Samples were rapidly filtered through GF/C filters using a Brandell Cell Harvestor, the filters were then washed with two 5-ml vol. of cold 10 mM $NaKPO_4$ at pH 7.4, and dried overnight, and the trapped radioactivity was counted in 8 ml of a toluene base scintillation mixture at an efficiency of 35–40%.

Results

Cholinergic agonists were observed to produce marked (>50%) shortening of AP duration in stimulated guinea pig right atrial strips (Fig. 1A); other parameters of the AP (resting potential, action potential amplitude, maximal upstroke velocity) were affected only minimally (see Table 1). The onset of the cholinergic effect was noted as early as 1 min after the start of drug superfusion, and the maximal effect was typically observed after 5–7 min. Oxotremorine was found to be the most potent, being approximately 6-fold greater than carbachol and more than 10-fold greater than acetylcholine. (The low potency for acetylcholine was

* Abbreviations: GTP, guanosine 5′-triphosphate; G_i, inhibitory guanine nucleotide binding protein; G_o, guanine nucleotide binding protein of unknown function; Gpp(NH)p, guanosine 5′-(βγ-imino)-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NEM, *N*-ethylmaleimide; and QNB, quinuclidinyl benzilate.

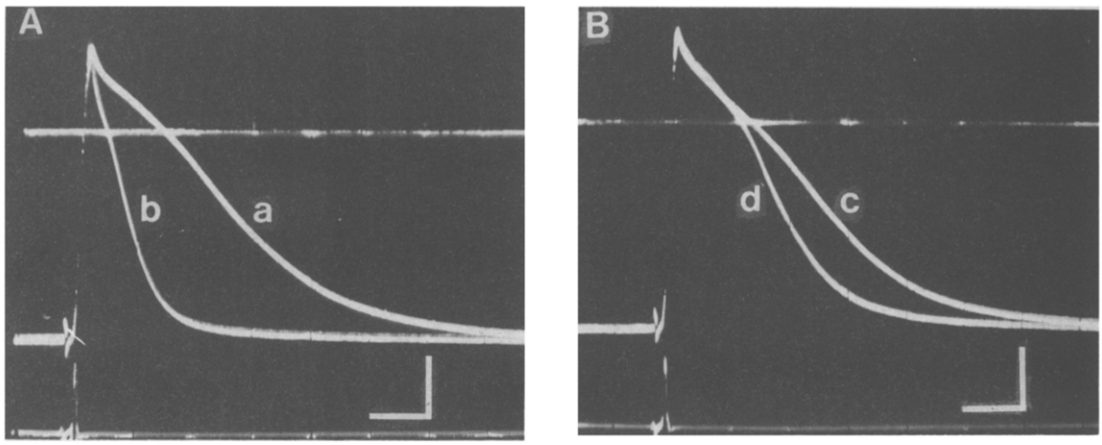

Fig. 1. Effects of NEM treatment on evoked action potentials in the right atrium. Representative action potentials are shown before (panel A) and after treatment with 100 μM NEM for 25 min (panel B) in both the absence (a, c) and presence (b, d) of 100 nM oxotremorine. The upper horizontal line indicates 0 mV; the lower trace is the first derivative of the AP upstroke. The vertical scale bar represents 20 mV for the AP traces and 100 V/sec for the first derivative trace respectively; the horizontal scale represents 20 msec. Recordings shown were all made from the same impalement and have been superimposed for illustrative purposes.

likely due to the absence of an acetylcholinesterase inhibitor in the perfusing solution.) The effects of cholinergic agonists were inhibited by low (50 nM) concentrations of the muscarinic antagonist atropine (results not shown). The β-adrenergic agonist isoproterenol moderately increased the AP amplitude, upstroke velocity, and duration (Table 1); these effects were reversed completely in the presence of equal concentrations of cholinergic agonists (data not shown).

Treatment of atrial strips with the -SH group alkylating agent NEM had little or no effect on the control AP parameters (Table 1, Fig. 1B), as shown by a comparison of the parameters prior and subsequent to NEM treatment. However, NEM exposure did cause significant inhibition of the cholinergically induced shortening of AP duration (Fig. 1B); the effects of oxotremorine, carbachol and acetylcholine were decreased by more than 50% of that observed prior to NEM exposure. NEM also decreased somewhat, but not significantly, the response to isoproterenol. Thus, these data indicate that NEM exposure selectively interferes with the hormone-induced changes in AP parameters.

In another series of experiments, NEM treatment (100 μM for 25 min) of rabbit atria was found to alter both oxotremorine binding to cholinergic receptors and the sensitivity of agonist binding to guanine nucleotides. (Rabbit right atria were observed to respond similarly to guinea pig right atria in preliminary microelectrode experiments; thus, their greater tissue yield proved more convenient for receptor binding characterization.) NEM decreased ($\approx$3-fold) agonist binding affinity (K_i), as indicated by the rightward shift in the [^{3}H](−)QNB/oxotremorine competitive displacement curve, and prevented the guanine nucleotide-induced decrease in agonist affinity, as observed by the weak rightward shift in the presence of the nonhydrolyzable GTP analogue, Gpp(NH)p (Fig. 2). Total receptor binding, as assessed by [^{3}H](−)QNB, was not affected by NEM treatment.

Discussion

The observed shortening of AP duration produced by cholinergic agonist is likely due to activation of a G protein-linked K^+ efflux [4–6], leading to a more rapid onset of AP repolarization. This differs from the observed electrophysiological effects of β-adrenergic stimulation, which are thought to be mediated by elevation of intracellular cyclic AMP [14].

The lack of effect of NEM treatment on control AP parameters indicates that the intrinsic action potential mechanisms are not compromised by NEM. Thus, the inhibition by NEM treatment of cholinergic attenuation of AP duration suggests that a component(s) in this transmembrane signalling process may be affected. While NEM could conceivably disrupt any one of the pathway components (i.e. hormone receptor, intermediary G-protein, K^+ channel complex), the observed disruption of cholinergic agonist binding by NEM strongly suggests that the G-protein/receptor interaction is primarily affected, since G-proteins are recognized to mediate both the agonist (but not antagonist) high affinity binding state and the effects of guanine nucleotides on agonist binding [15]. The lack of NEM effect on [^{3}H](−)QNB binding suggests that receptor binding properties *per se* are not compromised. These observations are in agreement with earlier findings [8, 16]. Furthermore, the recent demonstration [17] of direct NEM binding to the G-protein, G_o, provides additional support for the cholinergically associated G-protein(s) as the primary target of NEM treatment.

In a related study comparing the negative chronotropic and inotropic responses to carbachol in rat atria, NEM treatment (30 μM for 5–10 min) was found to inhibit only the cholinergically induced chronotropic response, the latter being affected only slightly [18]. These findings support the above observation that NEM antagonized primarily cholinergically stimulated AP shortening. That NEM treatment also did not affect the *basal* inotropic and chronotropic rates in rat atria agrees with the lack of NEM effect on control AP parameters observed in this study.

In summary, this study demonstrated that NEM pretreatment did not alter the parameters of evoked APs in guinea pig right atrium, but selectively attentuated the cholinergically induced shortening of AP duration. NEM treatment was also observed to decrease agonist binding affinity to atrial muscarinic cholinergic receptors and its regulation by guanine nucleotides. These findings suggest that NEM disrupts the integrity of the cholinergic signalling pathway in atrium by interfering with the function of regulatory G-proteins, possibly by alkylation of critical -SH groups.

Table 1. Effects of NEM treatment on adrenergic and cholinergic induced changes in AP parameters

	Before NEM treatment				After NEM treatment*			
	RP (mV)	APA (mV)	$\dot{V}_{max}$ (V/sec)	APD_{50} (msec)	RP (mV)	APA (mV)	$\dot{V}_{max}$ (V/sec)	APD_{50} (msec)
Control (N = 5)	−72.0 ± 1.3	100.0 ± 2.7	87.6 ± 19.7	57.4 ± 7.1	−72.0 ± 3.9	102.0 ± 4.7	85.6 ± 21.8	63.2 ± 10.1
Drug	% Change from control							
Oxo (N = 4)	+1.4 ± 3.5	−5.0 ± 6.0	−4.3 ± 4.7	−60.1 ± 11.7	+2.1 ± 1.5	−3.7 ± 3.7	−1.5 ± 2.6	−22.2 ± 8.1†
Cch (N = 2)	−2.8 ± 1.4	−5.0 ± 1.0	−5.7 ± 0.6	−62.7 ± 8.7	0 ± 0.7	−2.5 ± 1.5	0 ± 0.6	−24.5 ± 11.9
Ach (N = 2)	+0.7 ± 0.7	+0.5 ± 0.5	+2.9 ± 2.9	−26.1 ± 3.5	−1.4 ± 1.4	+1.0 ± 0.5	+2.9 ± 2.9	−4.0 ± 2.4
Iso (N = 3)	−1.0 ± 1.7	+3.7 ± 1.2	+4.9 ± 1.0	+23.9 ± 9.4	+2.4 ± 4.3	−1.0 ± 2.7	0 ± 4.8	+13.3 ± 0.8

Control values are given as absolute values (±SEM). The drug effects are expressed as the percent change (±SEM) from the control value at the top of the column, either increase(+) or decrease(−), from the washout control value observed immediately preceding the application of drug. Changes in resting potential are given as either hyperpolarization (+) or depolarization (−). Drug concentrations among experiments were as follows: oxotremorine (Oxo), 25–100 nM; carbachol (Cch), 150 nM; acetylcholine (Ach), 250–500 nM; isoproterenol (Iso), 25–100 nM. Abbreviations: RP, resting potential; APA, action potential amplitude; $\dot{V}_{max}$, maximal upstroke velocity; APD_{50}, action potential duration at 50% repolarization.

* NEM exposure was either 100 μM for 25 min or 50 μM for 40 min.

† Statistical comparison using Student's unpaired *t*-test was made between non-normalized pre- and post-NEM treatment values, $P < 0.013$.

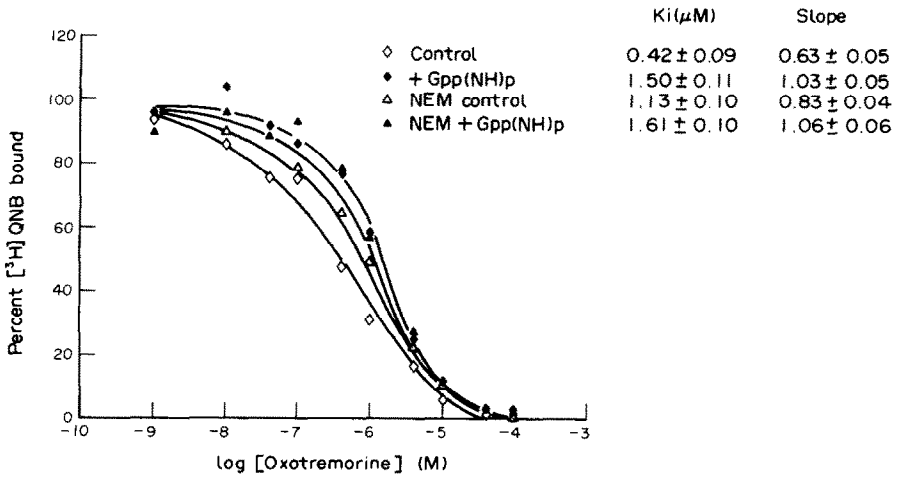

Fig. 2. Effects of NEM treatment on guanine nucleotide sensitive oxotremorine binding in rabbit atria. Oxotremorine binding in control and NEM-treated atria (see Methods) was assessed in the absence and presence of 100 μM Gpp(NH)p; binding data were analyzed by the curve-fitting program EBDA [12]. K_i (μM) and slope represent the corrected IC_{50} value and Hill coefficient respectively. To obtain the K_i value, 27 pM was used as the estimate of the [^{3}H](−)QNB dissociation constant [13]. Curves are representative data from a typical experiment.

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Inhibition of the formation of 4-hydroxyandrostenedione glucuronide by valproate

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The steroid, 4-hydroxyandrostenedione (HAD) is a potent specific inhibitor of the estrogen synthetase enzyme complex, aromatase which is responsible for the conversion of androgens into estrogens [1, 2]. Clinical trials have shown HAD to be capable of suppressing plasma estradiol levels and producing tumour regression in postmenopausal patients with advanced breast cancer [3, 4]. Metabolism studies demonstrated that HAD is rapidly conjugated to the glucuronide and this is the principal metabolite in rats and humans [5, 6]. Extensive conjugation of HAD has been proposed as the reason for the inability to detect HAD in rat plasma using gas chromatography–mass spectrometry, despite a sensitivity down to 50 ng/ml [5]. The present investigation is an attempt to reduce the extent of this "adverse" metabolism. Taburet and Aymard [7] have shown that the drug, valproate (VPA), inhibits the glucuronide conjugation of parahydroxyphenobarbital by rat liver microsomes. Experiments are described below in which the effect of VPA on the conjugation of HAD is studied using rat hepatocytes. Plasma HAD levels and amounts of HAD glucuronide in bile were estimated in rats given HAD alone and HAD + VPA.

Materials and methods

Materials. [^{14}C]4-hydroxyandrostenedione was synthesized as described by Foster *et al.* [6]. Valproic acid and cyclohexane carboxylic acid were obtained from Aldrich Chemical Co Ltd (Gillingham, U.K.). For *in vitro* experiments valproic acid was neutralized with N NaOH to form sodium valproate whilst for the *in vivo* work sodium valproate was given in the form of a syrup, Epilim, containing 40 mg/ml. β-Glucuronidase, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. Lipidex 5000™ and Sep-Pak G_{18} cartridges were obtained from Canberra Packard and Waters Assoc. (Milford, MA) respectively.

In vitro *studies.* Hepatocytes were isolated from the livers of phenobarbital treated male Wistar rats by a 2-step collagenase perfusion technique previously described [6]. Suspensions of hepatocytes (5×10^6 cells/ml) were incubated with 0.33 mM HAD labelled [6] with [^{14}C] (208 μCi/mmole) in the presence or absence of various concentrations of valproate (0.75 mM–3 mM). Samples of incubation mixture were extracted and processed as described [6] and finally the amounts of Phase I and Phase II metabolites determined after separation by thin layer chromatography and estimation of the relative proportion of radioactivity in each band using a Berthold TLC Linear Analyzer [6]. Identification of Phase I and Phase II metabolites of HAD has been described [6].

In vivo *studies.* Drugs were administered to the rats by gastric gavage, either 2.5 mg HAD in standard steroid vehicle or 2.5 mg HAD with varying amounts of valproate syrup containing 12.5–50 mg of sodium valproate. The rats were bled by cardiac puncture 1.5 hr later. For the 24 hr bile collection, the bile duct was cannulated under pentobarbitone anaesthesia immediately after HAD or HAD plus valproate were given orally.

Isolation of HAD from plasma. The plasmas (minimum vol of 2 ml) were passed through Sep-Pak cartridges and the dried eluates further purified by Lipidex column chromatography. Lipidex 5000 pre-equilibrated in 2,2,4-trimethylpentane : isopropanol (5:1) (TMP:IP) was packed into a glass column 420 mm × 4 mm. The elution profiles of androstenedione and HAD were pre-determined for each column using [^{3}H]androstenedione and [^{14}C]HAD. The dried eluate from the cartridges was dissolved in the solvent system and applied to the column and the appropriate fractions equivalent to HAD collected.

Isolation of HAD glucuronide from bile. Samples (24 hr) of bile were extracted with ethyl acetate at pH 1.5 to give a glucuronide fraction (mostly HAD glucuronide) which was hydrolysed with β-glucuronidase and the HAD so released estimated using the aromatase assay.

Estimation of HAD by aromatase assay. The pooled fractions containing HAD were transferred to assay tubes, taken to dryness and resuspended in 25 μl of ethanol. Activity of the aromatase enzyme from human placental microsomes was monitored by measuring the tritiated water formed from [^{3}H]androstenedione in the presence of a NADPH regenerating system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase [8]. Each sample was assayed in duplicate over three time points. The results were plotted on a graph of product released against time of incubation. The resulting linear graph was utilised to determine the rate of enzymic reaction for each sample. The values were compared to control samples (normal plasma) and the per cent activity of control samples determined. A calibration curve (per cent activity of aromatase enzyme against concentration of HAD) was produced using normal plasma containing a known concentration of HAD and working up the samples as described. Quantification of HAD present in samples of unknown concentration was made by comparison of inhibitory activity with the calibration curve. The lower limit of sensitivity of this bioassay was 10 ng of HAD/ml.

Results and discussion

Addition of VPA to a suspension of rat hepatocytes metabolising [^{14}C]HAD caused a fall in the amount of radioactive label appearing in the conjugate-containing aqueous fraction whilst at the same time levels of [^{14}C] in the ethyl acetate fraction (un-metabolised HAD and phase I metabolites) were increased (Table 1). Further analysis of radiochromatograms (not shown) from this latter fraction revealed that although only small amounts of HAD