The effects of cocaine on intracellular Ca^{2+} handling and myofilament Ca^{2+} responsiveness of ferret ventricular myocardium

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1 When ferret right ventricular papillary muscles were stimulated with threshold punctate pulses (0.33 Hz; 30°C), cocaine, 10^{-5} M, increased peak tension development from 815 ± 120 to 1125 ± 180 mg ($P < 0.05$) and increased the rate of relaxation from peak tension (time to 80% decline from peak tension decreased from 155 ± 11 to 144 ± 11 ms; $P < 0.05$). These changes in the twitch were associated with comparable changes in the amplitude and time course of the calcium transient measured with aequorin (amplitude increased from 62 ± 4 to $90 \pm 7\%$ ($P < 0.05$) of maximal values; time to 80% decline from peak amplitude decreased from 84 ± 8 to 64 ± 3 ms; $P < 0.05$). These effects were markedly attenuated in the presence of the β -adrenoceptor-blocking agent, propranolol, 6×10^{-7} M, or by maximization of catecholamine release from the adrenergic nerve endings with field pulses of suprathreshold strength, indicating that catecholamine release from the adrenergic nerve endings is responsible for the positive inotropic and lusitropic responses to low and moderate doses of cocaine (i.e., $\leq 10^{-5}$ M).

2 High doses of cocaine (i.e., $> 10^{-5}$ M) produced negative inotropic and lusitropic effects that were associated with a decreased amplitude and prolonged duration of the calcium transient.

3 In aequorin-loaded intact fibres, cocaine 10^{-5} M did not affect the force-calcium relationship unless catecholamines were present. Cocaine, 10^{-5} M, significantly shifted the force-calcium relationship of saponin-skinned muscles ($\text{pCa}_{50} = 6.14 \pm 0.05$ versus 5.92 ± 0.07 ; $P < 0.05$), indicating reduced responsiveness of the myofilaments to calcium. F_{max} (maximal Ca^{2+} -activated force) was reduced to 58% of control in the presence of 10^{-5} M cocaine, while the slope of the calcium-force curve remained unchanged. These data indicate that cocaine may also decrease myofilament calcium sensitivity and maximal calcium-activated force, via mechanisms independent of catecholamines, when cellular diffusion barriers are eliminated.

Introduction

The past several years have witnessed a dramatic increase in the non-medical use of cocaine and the advent of 'free-basing', which introduces large quantities of active drug into the bloodstream. Consequently, the incidence of drug-related cardiovascular complications is on the increase in Europe and the United States and now accounts for a significant fraction of cocaine-related disability and death. There are five clinically documented cardiac and vascular complications of cocaine use and abuse: (1) coronary and peripheral vascular spasm with regional ischaemia of the heart and mesenteric viscera, (2) myocardial infarction, (3) hypertension and stroke, (4) dysrhythmia and sudden cardiac death, (5) myocarditis, dilated cardiomyopathy and heart failure. Although the pathogenesis of the cardiovascular toxicity of cocaine remains to be defined, available evidence suggests that cocaine has medical consequences that are equal in importance to its well-documented psychosocial consequences (Nicholi, 1983; Isner *et al.*, 1986; Smith *et al.*, 1987).

Because calcium is an essential second messenger in excitation-contraction coupling of both the heart and blood vessels, the major cardiovascular toxicity of cocaine may be due to drug-induced alterations in intracellular Ca^{2+} handling and possibly the development of a Ca^{2+} overload state (Reiter, 1988; Morgan *et al.*, 1990). Depending upon the species studied and the precise experimental conditions, cocaine has been shown to have a positive inotropic, negative inotropic, or biphasic inotropic effect (Covino, 1986). In general, positive inotropic actions of this drug appear to correlate with increased catecholamine levels related to cocaine-induced blockade of the Uptake₁ system of the adrenergic nerve endings (Muscholl, 1961; Burgen & Iversen, 1965;

Iversen, 1971). In contrast, negative inotropic actions appear to correlate with the local anaesthetic effects of higher concentrations of cocaine (Covino, 1986). In many experimental and clinical situations, the effects of cocaine are mixed and appear to involve a combination of these two mechanisms. In addition, direct actions on the sodium-calcium exchange mechanism and on the calcium pump of the sarcoplasmic reticulum have also been proposed for cocaine or related local anaesthetic agents (Blinks *et al.*, 1972; Josephson & Sperelakis, 1976).

The purpose of the present study was to test the hypothesis that the positive and negative inotropic actions of cocaine are related to changes in intracellular Ca^{2+} handling (Hague *et al.*, 1988; Perreault *et al.*, 1989). We also investigated, in skinned ferret ventricular muscles, the effects of cocaine on myofilament calcium responsiveness, which includes calcium sensitivity, maximal calcium-activated force and the slope of the calcium-force relationship (Perreault *et al.*, 1990a).

Although a wide range of blood levels has been obtained in conjunction with the cardiovascular actions or toxicity of cocaine in man, values usually range from between 10^{-6} to 5×10^{-5} M (Van Dyke *et al.*, 1976; Connor & Macleod, 1976; Wetli & Wright, 1979; Paly *et al.*, 1982; Isner *et al.*, 1986; Foltin *et al.*, 1988). On this basis, we selected a cocaine concentration of 10^{-5} M for use in the majority of our experiments.

Methods

Tissue acquisition and initial preparation

Papillary muscles of 1.0 mm or less in diameter were excised from the right ventricles of hearts removed from adult male ferrets, 12–14 weeks of age, under chloroform anaesthesia. The methods of preparation and instrumentation used in these

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studies have been described in detail (Gwathmey & Morgan, 1985; McKinnon *et al.*, 1988; Kihara *et al.*, 1989). After removal from the hearts, muscles were placed in baths containing bicarbonate-buffered physiological salt solution bubbled with a gas mixture of 95% O₂ and 5% CO₂ to pH 7.4. The experiments were performed at 30°C. Muscles were stimulated to contract at three second intervals with pulses of 5 ms duration. Unless otherwise specified, threshold voltage (i.e., $\leq 10\%$ above threshold) delivered via punctate or field electrodes was used to stimulate the muscles; under some circumstances, maximal field stimulation was used in order to maximize catecholamine release from the adrenergic nerve endings (Blinks, 1966). Pulses were applied through either punctate or field electrodes located at the base of the muscle. An initial two hour equilibration period was allowed during which muscles were gradually stretched to the length where maximal isometric force developed. Since it was possible to obtain up to four papillary muscles per ferret, our *n* refers to the number of papillary muscles, not the number of ferrets.

Intact muscle studies

One group of muscles (*n* = 9) was used to characterize the actions of chlorobutanol, atropine and propranolol under conditions of threshold punctate or threshold field stimulation. In another group of punctate stimulated muscles (*n* = 16), cumulative concentration-response relationships for cocaine were determined. Tension, time to peak tension, and time from peak tension to 50% and 80% decline from peak tension were measured on chart strip paper which simultaneously recorded the tension response and the stimulus artifact at 100 mm s⁻¹. All measures were made under steady state conditions. In order to avoid precipitation of Ca²⁺ during the cumulative calcium concentration-response determinations, a phosphate-free solution was used and calcium was added in incremental concentrations up to 16 mM.

In a third group of muscles (*n* = 7 threshold punctate; *n* = 7 threshold field and maximal field), aequorin was loaded by macroinjection, as described in detail elsewhere (Kihara & Morgan, 1989). Light signals were recorded with a photomultiplier by means of a light collecting apparatus of a design described by Blinks (1982). In order to obtain a satisfactory signal-to-noise-ratio, it was usually necessary to average successive signals (from 16 to several hundred depending on the light intensity). Signal averaging was performed only after responses had reached the steady state. The light and tension responses and stimulus artifact were recorded simultaneously both on magnetic tape and on chart strip recording paper; light signals were passed through a filter with a 10 ms time constant. The light signal was measured in nanoamperes of anodal current. The amplitude and time course of the aequorin light signal were analysed in the same manner described above for tension. It is possible for drugs to interact directly with aequorin and modify the luminescent reaction or the sensitivity of aequorin to Ca²⁺ (Kamaya *et al.*, 1977; Baker & Schapira, 1980; Endoh & Blinks, 1988). Therefore, the potential for interaction of cocaine and aequorin was tested *in vitro* by use of the basic method and calibration device described by Endoh & Blinks (1988). Briefly, aequorin was added to a solution containing free [Ca²⁺] between 10⁻⁷ and 10⁻⁶ M; under these conditions a low level of luminescence persists for several minutes until the aequorin is gradually consumed. After initiation of the luminescence reaction, cocaine dissolved in an aqueous diluent containing 0.5% chlorobutanol (see below), was added to the reaction pipette. We found that the drug had no effect on the luminescence reaction until concentrations in excess of 10⁻⁴ M were achieved. In these concentrations, the combination of cocaine plus aequorin appeared to decrease the light emission.

All data were compared by unpaired or paired (when appropriate) Student's *t* test or multiple sample comparison tests. Statistical significance was set at *P* < 0.05.

Skinned muscle studies

Muscles (*n* = 18) were skinned by a 30 min exposure to a solution containing: saponin 250 µg ml⁻¹, K₂ATP 5 mM, MgCl₂ 7 mM EGTA 5 mM KCl 60 mM imidazole 60 mM, creatine phosphate 12 mM and creatine phosphokinase 15 u ml⁻¹; pH = 7.1 at 21°C. Saponin, in this concentration, has been shown to remove functionally both the sarcolemma and sarcoplasmic reticulum (Endo & Iino, 1980). Total salt concentrations needed to obtain the desired pCa, pMg, pMgATP and pH at a constant ionic strength of 0.16 M were calculated using a program developed by Fabiato & Fabiato (1979). The absolute stability constants used were as described by Fabiato (1981). Muscles were subjected to activation-relaxation cycles which consisted of increasing the calcium concentration in a stepwise fashion until no further increase in force was produced, i.e., maximal calcium-activated force (*F*_{max}).

The isometric force versus pCa ($-\log[\text{Ca}^{2+}]_{\text{M}}$) data were fitted to a modified Hill equation. Values of pCa₅₀ (the pCa that produced 50% of maximal force development), *F*_{max} and *n* (the slope of the calcium-force curve) were compared by analysis of variance and Student's *t* tests. Statistical significance was set at *P* < 0.05.

Drugs

The aequorin used in these experiments was obtained from the laboratory of Dr J.R. Blinks of Rochester, Minnesota, U.S.A.; propranolol was purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. and dissolved in aqueous solution. A stock solution of cocaine, 10⁻¹ M, (purchased from Mallinckrodt, Inc., St. Louis, MO, U.S.A.) was obtained from the Beth Israel Hospital Pharmacy dissolved in a diluent containing 0.5% chlorobutanol. In the volumes added to the bath in these experiments, the diluent had no effect on the amplitude or time course of muscle contraction (see below).

Results

Effect of stimulus mode

Table 1 and Figure 1 show the relative effects of three different stimulus modes on the amplitude and time course of the intracellular calcium transient ([Ca²⁺]_i), recorded with aequorin, and the isometric tension response. Note that threshold punctate and field stimulation produced similar peak-tension responses. In contrast, maximal field stimulation (the voltage that produced maximal activation) produced a significantly greater amount of tension development. Previous work has shown that the maximal response to field stimulation is equivalent to that produced by maximally effective concentrations of exogenously applied noradrenaline (Blinks, 1966). Table 1 also demonstrates the positive lusitropic (i.e., enhanced rate of relaxation) effect that occurred with threshold and maximal field stimulation compared to the punctate modality. Note that maximal field stimulation markedly abbreviated the duration of the twitch and enhanced relaxation, while threshold field stimulation produced a trend in that direction which did not reach statistical significance, except in the case of the time to 80% relaxation from peak tension (t8t).

Peak [Ca²⁺]_i was not measured in these experiments, and the peak aequorin light signals could not be accurately compared between different experiments due to variable degrees of aequorin loading and consequent muscle brightness; therefore, no value for peak [Ca²⁺]_i could be included in Table 1. However, Table 1 demonstrates that maximal field stimulation significantly abbreviated the time course of the Ca²⁺ transient, which correlates with the positive lusitropic response to this mode of stimulation.

Figure 1 shows the effect of the stimulation mode on the inotropic response to cocaine. Note that in both the punctate

Table 1 Effect of stimulus parameters on amplitude and time course of the aequorin light signal and tension

	Punctate (threshold V) (n = 16)	Field (threshold V) (n = 7)	Field (maximal V) (n = 7)
Pt (mg)	815 ± 120	760 ± 134	1951 ± 334* #
tPt (ms)	179 ± 10	162 ± 7	124 ± 4* #
t5t (ms)	101 ± 7	88 ± 8	59 ± 4* #
t8t (ms)	155 ± 11	72 ± 5 #	87 ± 4* #
tPL (ms)	54 ± 3 (n = 7)	54 ± 2	41 ± 2* #
t5L (ms)	46 ± 2 (n = 7)	43 ± 2	30 ± 2* #
t8L (ms)	84 ± 8 (n = 7)	70 ± 5	49 ± 2* #

Data shown are means ± s.e.mean.

# $P < 0.05$ versus punctate; * $P < 0.05$ versus threshold field; Pt = peak tension; tPt = time to Pt, t5t and t8t = time to 50% and 80% decline from peak tension, respectively; tPL = time to peak light; t5L and t8L = time to 50% and 80% decline from peak light, respectively. Note that tPL and tPt were measured from stimulus artifact and t5L, t5T, t8L and t8T from peak response. Muscles were bathed in 1 mM calcium at 30°C and stimulated at 3 s intervals. V = voltage of 5 ms duration pulses.

and field modes, when threshold voltage was utilized, cocaine 10^{-5} M produced a significant positive inotropic effect. In contrast, when this concentration was administered to muscles field stimulated with a maximally effective voltage, no signifi-

cant change was observed, although a negative inotropic trend was present in some experiments. In view of these responses, we used threshold stimulation to evaluate the sympathomimetic actions of cocaine.

Table 2 shows the effects of several bath additives on the amplitude of contraction in muscles stimulated with threshold voltage via field electrodes ($n = 9$). First, it should be noted that chlorobutanol in the amounts used in these experiments produced no significant effect on the contractile state. Under control conditions, cocaine 10^{-5} M, produced a statistically significant positive inotropic effect. Atropine, 2×10^{-6} M also increased the force of contraction of the preparations, an effect that was reversed by the subsequent addition of propranolol, 6×10^{-7} M. Table 2 demonstrates the dependence of the positive inotropic action of cocaine on the presence of functionally intact cardiac autonomic receptors. In the presence of atropine plus propranolol, the inotropic response to cocaine, 10^{-5} M, was markedly attenuated.

Effects of cocaine on the amplitude of the aequorin light signal and tension response

Figure 2 shows the effects of cocaine, 10^{-5} M, on the intracellular $[Ca^{2+}]_i$ transients recorded with aequorin and isometric contractions of a ferret papillary muscle. In this experiment the muscle was stimulated to contract with threshold field pulses. Note that this concentration of cocaine produced a marked increase in $[Ca^{2+}]_i$ that was associated with an increase in developed force. Table 3 illustrates these effects for 3 different concentrations of cocaine, applied to muscles being stimulated to contract with threshold voltage delivered via punctate electrodes. Note that 10^{-6} and 10^{-5} M cocaine produced similar increases in peak tension to levels that were significantly greater than the control. These increases in tension were associated with parallel increases in the amplitude of the intracellular Ca^{2+} transient. Higher concentrations (i.e., 10^{-4} M) of cocaine produced a negative inotropic effect that was associated with a decrease in the amplitude of the aequorin signal, reflecting decreased availability of $[Ca^{2+}]_i$ for excitation-contraction coupling.

Figure 1 Dependence on stimulus parameters of peak tension development and inotropic responses to cocaine, 10^{-5} M, of ferret right ventricular papillary muscles. Open columns represent control responses in absence of cocaine and stippled columns, responses in presence of 10^{-5} M cocaine. Values shown are means and bars indicate s.e.mean. * $P < 0.05$ versus control.

Table 2 Effects of bath additives on amplitude of contraction of ferret papillary muscles stimulated with threshold field voltage

	Diluent #	Cocaine (10^{-5} M) W	Atropine (2×10^{-6} M) W	Atropine (2×10^{-6} M) + propranolol (6×10^{-7} M)	Atropine (2×10^{-6} M) + propranolol (6×10^{-7} M) + cocaine (10^{-5} M)
% change	-1.5 ± 1.9%	+21 ± 7.7%*	+16.6 ± 2.6*	-14 ± 6*	+6.8 ± 4.5*

Data shown are means ± s.e.mean, $n = 9$.

* $P < 0.05$ versus previous steady state value.

-, indicates decrease; +, increase from control values.

Cocaine vehicle containing 0.5% chlorobutanol. W indicates washout.

Figure 2 Effects of cocaine on a ferret right ventricular papillary muscle stimulated to contract at 3 s intervals with threshold field pulses of 3 V, 5 ms duration. Upper trace, aequorin light signal expressed in nanoamperes of anodal current from photomultiplier; middle trace, isometric force in g, lower trace, stimulus artifact. Forty successive signals were averaged under steady-state conditions to obtain these aequorin signals.

Effects of cocaine on the time course of the light signal and isometric contraction

Table 3 shows that low and moderate concentrations of cocaine (i.e., 10^{-6} and 10^{-5} M, respectively) significantly abbreviated the mechanical twitch, as reflected by the time to 80% relaxation from peak tension (t8t), and the corresponding Ca^{2+} transient, as reflected by the time to 80% decline from peak light (t8L). Cumulative additions of higher concentrations of cocaine (i.e., 10^{-4} M) tended to prolong both the twitch and calcium transient back towards control levels (although this change reached significance only for the time to peak light).

Effects of cocaine on myofilament Ca^{2+} responsiveness

In order to determine what effect, if any, cocaine has on myofilament Ca^{2+} responsiveness, we evaluated the force versus pCa relationship in aequorin-loaded fibres by determining the Ca^{2+} sensitivity index from the light versus force relationship in the absence and presence of the drug. This relationship can be expressed as the ratio of the amplitude of the light signals at equi-inotropic concentrations of drug and $[\text{Ca}^{2+}]_0$, i.e., peak light (cocaine) $\div$ peak light ($[\text{Ca}^{2+}]_0$), expressed in nanoamperes. In muscles stimulated with threshold punctate pulses, low doses of cocaine had no significant effect on this ratio, producing light signals (i.e., intracellular Ca^{2+} transients) with equivalent amplitudes to those of equi-inotropic $[\text{Ca}^{2+}]_0$ (sensitivity index = 0.9 ± 0.1 , $n = 11$). Concentrations of cocaine in excess of 10^{-5} M tended to increase this ratio in a direction suggesting decreased Ca^{2+} responsiveness, although this effect did not achieve statistical significance (data not shown). In contrast, when cocaine 10^{-5} M was added to the bathing medium of muscles being stimulated with maximal field pulses, the ratio increased to 1.7 ± 0.5 , $n = 5$, ($P < 0.05$ versus punctate), suggesting that decreased Ca^{2+} responsiveness can occur at moderate concentrations of

cocaine when catecholamine release is maximally facilitated. This effect of cocaine on maximal field stimulated muscles did not occur in the presence of propranolol, 6×10^{-7} M (sensitivity index = 0.9 ± 0.1 , $n = 5$).

To evaluate more directly the effects of cocaine on myofilament Ca^{2+} responsiveness, which includes calcium sensitivity, maximal calcium activated force and the slope of the calcium-force relationship, we examined the effect of the drug on skinned muscle preparations. Cocaine, 10^{-5} M, decreased the responsiveness of the myofilaments to calcium in saponin-skinned ferret ventricular myocardium ($n = 18$). As shown in Figure 3, the sigmoidal force versus calcium curve was significantly shifted to the right in the presence of 10^{-5} M cocaine (6.14 ± 0.05 versus 5.92 ± 0.07 , $P < 0.05$), thus indicating a decreased sensitivity of the contractile proteins to calcium (Figure 3). However, the Hill coefficient, which reflects the slope of the fit at the level of half maximal Ca^{2+} activation, was not altered by cocaine (1.28 ± 0.02 versus 1.25 ± 0.03 before and after cocaine 10^{-5} M, respectively). Maximal calcium-activated force, the maximal amount of force that a muscle can generate in the presence of calcium, was significantly decreased in the presence of 10^{-5} M cocaine to $58 \pm 7.5\%$ of the control level ($P < 0.05$).

Discussion

Interpretation of aequorin light signals in heart muscle

As shown in Figure 2 for ferret papillary muscles, the aequorin signals from mammalian working myocardium consist of a single component that temporally precedes the corresponding tension response. These findings are consistent with current models of excitation-contraction coupling, which predict that mechanical contractile events are preceded by changes in intracellular calcium concentrations (Rüegg, 1988). Interpreta-

Figure 3 Effects of cocaine on saponin-skinned muscles. Maximal force was normalized and expressed as 100% for each muscle. (●—●) Control; (○---○) effect of cocaine 10^{-5} M. Values shown are means and vertical lines indicate s.e. * $P < 0.05$ versus control.

Table 3 Effects of cocaine on aequorin loaded right ventricular papillary muscles stimulated with threshold punctate voltage

	Control	10^{-6} M Cocaine	10^{-5} M Cocaine	10^{-4} M Cocaine
Peak tension (mg)	815 ± 120	$1122 \pm 174^*$	$1125 \pm 180^*$	$674 \pm 127^*$
Peak light (% max Ca^{2+} ; $n = 7$)	62 ± 4	$94 \pm 6^*$	$90 \pm 7^*$	40 ± 11
tPL (ms; $n = 7$)	54 ± 3	50 ± 0	49 ± 1.5	$61 \pm 7^*$
tPt (ms)	179 ± 10	$167 \pm 8^*$	166 ± 7	164 ± 10
t5t (ms)	101 ± 7	96 ± 8	$95 \pm 8^*$	96 ± 8
t8t (ms)	155 ± 11	$147 \pm 12^*$	$144 \pm 11^*$	148.5 ± 11
t5L (ms; $n = 7$)	46 ± 2	$36 \pm 2^*$	$40 \pm 0^*$	41 ± 6
t8L (ms; $n = 7$)	84 ± 8	$61 \pm 3^*$	$64 \pm 3^*$	64 ± 6

Data shown are means $\pm$ s.e.mean, $n = 16$ unless indicated otherwise. % max Ca^{2+} = % of maximal response to Ca^{2+} . t5L = time to 50% decline from peak light; t8L = time to 80% decline from peak light; t5t = time to 50% decline from peak tension; t8t = time to 80% decline from peak tension; tPt = time to peak tension; tPL = time to peak light.

* $P < 0.05$ compared to control. All muscles were stimulated with threshold voltage via punctate electrodes at 0.33 Hz and bathed in 1 mM Ca^{2+} at 30°C.

tion of the aequorin light signals recorded from mammalian working myocardium have been detailed elsewhere (Morgan *et al.*, 1984; Morgan & Morgan, 1984; 1989). In brief, the aequorin signal in mammalian working myocardium appears to reflect predominantly the release and uptake of Ca^{2+} from the sarcoplasmic reticulum. Therefore, the peak of the aequorin light signal can be used as a measure of the amount of Ca^{2+} released by the sarcoplasmic reticulum. The time to peak of the aequorin light signal appears to reflect the relative balance between the phase of the Ca^{2+} transient during which Ca^{2+} release predominates versus the phase during which resequestration of Ca^{2+} predominates (descending phase). The half-time of decline from the peak of the aequorin light signal can be used as a measure of the speed of Ca^{2+} re-uptake by the sarcoplasmic reticulum. These interpretations are undoubtedly over-simplifications of the complex sequence of events that occur during excitation-contraction coupling in the heart, but are consistent with our current understanding of Ca^{2+} movement in myocardial cells. Determination of the relative effects of drugs on the amplitude of the aequorin light signal at similar levels of tension development provides an index for changes in the sensitivity of the contractile apparatus to Ca^{2+} ; an index that generally correlates well with results from experiments in skinned cardiac muscle fibres (Gwathmey & Morgan, 1985; Morgan & Morgan, 1984; 1989).

Effects of autonomic agents and specific stimulus modalities on ferret myocardium

In this regard, it is worth noting that muscarinic agonists such as carbachol inhibit the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent positive inotropic effects of drugs that act by increasing intracellular cyclic AMP levels. This muscarinic action presumably occurs through blockade of the inhibitory effect of cholinergic agonists on adenylate cyclase activity, which is mediated through activation of an inhibitory guanine nucleotide regulatory protein. In dog and human myocardium, this muscarinic effect is not observed unless cyclic AMP levels are raised above those present with threshold stimulation alone (Endoh *et al.*, 1986; Warren *et al.*, 1989).

It is of interest that the effects of atropine and propranolol in our ferret muscle preparations, under conditions of threshold stimulation, were significantly greater than those obtained for similar concentrations of these autonomic receptor blocking agents in guinea-pig atria and atria and papillary muscles of kittens. In these species, concentrations of atropine or propranolol sufficient to prevent or greatly diminish the parasympathomimetic or sympathomimetic effects of maximal field stimulation had virtually no effect on the strength of contraction of preparations driven with threshold stimuli (Blinks, 1966). These results suggest a much greater dependence of ferret ventricular muscle on intracellular cyclic AMP levels for modulation of contractile function under basal conditions. These results also indicate that the data of studies in which ferret papillary muscles are driven electrically must be interpreted in light of the strong likelihood that liberation of autonomic transmitters by the stimuli is appreciable, even under conditions where threshold punctate stimulation is employed. Therefore, a combination of threshold stimulation plus β -adrenoceptor blockade must be employed to unmask the direct effect of cocaine on the sarcolemmal Na^+ channels; such experiments are complicated by the additive effect of local anaesthetic drugs. Consequently, in experiments where we wished to minimize catecholamine release to as large an extent as possible threshold stimulation without addition of other agents was employed alone. As shown in Table 1, threshold field stimulation of ferret papillary muscles produced effects intermediate between threshold punctate and maximal field stimulation, but which were more similar to the former than the latter. These effects of threshold stimulation are also presumably related to a greater quantity of catechol-

amine released from the larger number of sympathetic nerve endings which are affected by the field (versus punctate) electrodes.

Effects of cocaine on amplitudes of the aequorin light signal and tension responses

The amplitude of the aequorin light signal in ferret working myocardium is increased by most positive inotropic drugs, including isoprenaline, dibutyl cyclic AMP, isobutylmethylxanthine, forskolin and increases in extracellular $[\text{Ca}^{2+}]$. Similar findings have been noted in other mammalian species (Morgan & Morgan, 1984; 1989; Perreault *et al.*, 1990a). These results are consistent with the expectation that an increased concentration of intracellular calcium would be reflected at the level of the myofilaments by an enhanced degree of activation. Agents like tyramine, amphetamine and bretylium produce a positive inotropic effect that is mediated by noradrenaline released from adrenergic nerve endings (Muscholl, 1961; Burgen & Iversen, 1965; Iversen, 1971). It is reasonable to hypothesize, therefore, that the positive inotropic effect of cocaine would be mediated by similar mechanisms.

As shown in Table 3, low concentrations of cocaine produce positive inotropic and lusitropic responses that are associated with an increase in amplitude and abbreviation of the duration of the Ca^{2+} transient. Moreover, when catecholamine release is facilitated, the ratio of the peak light responses at equi-inotropic concentrations of cocaine and $[\text{Ca}^{2+}]_i$ is increased in a direction suggesting desensitization of the contractile apparatus to calcium. This combination of effects is characteristic of agents that act by increasing intracellular cyclic AMP concentrations (Morgan & Morgan, 1989).

Phosphorylation of the Ca^{2+} -regulatory protein, phospholamban, results in a markedly increased rate of calcium resequestration by the calcium pump (Katz, 1979). This is reflected in the aequorin signal by an increased rate of decline (i.e., decreased t5L and t8L as shown in Table 3) and by a positive lusitropic effect as reflected by the decreased t5t and t8t (see Table 3). Moreover, phosphorylation of the myofilaments themselves, in particular, the troponin-C regulatory subunit, results in a decreased myofilament calcium sensitivity. This is reflected in aequorin-loaded fibres by the rightward shift which occurs in the ratio of the peak light responses at equi-inotropic concentrations of cocaine and $[\text{Ca}^{2+}]_o$. In the presence of low concentrations of cocaine, a much larger Ca^{2+} transient is required to produce an equi-inotropic response compared to control conditions. Therefore, the combination of effects noted at concentrations of cocaine $\leq 10^{-5}$ M are consistent with the actions of β -adrenoceptor stimulation, due to increased noradrenaline content in the synaptic cleft and biophase caused by cocaine-induced blockade of the Uptake₁ system of the adrenergic neurones. Additional substantiation of this interpretation is provided by attenuation of these responses when catecholamine effects are minimized via the use of threshold stimulation, and by the marked attenuation noted in the presence of propranolol (Table 2).

Biphasic action of cocaine

Higher concentrations of cocaine produce opposite effects to those described above. Concentrations of 10^{-4} M not only decreased the amplitude of the light and tension responses but also tended to prolong the time course towards control levels. These effects are probably related to the local anaesthetic actions of cocaine, since they were not affected by changes in the strength of field stimulation or altered by the addition of atropine. Local anaesthetics decrease the amount of Na^+ that enters the sarcoplasm via the voltage-dependent sarcolemmal Na^+ channel and could thereby decrease the amount of Ca^{2+} entering the cell via the Na^+ - Ca^{2+} exchanger (Reiter, 1988). This in turn would be expected to decrease Ca^{2+} loading at

the sarcoplasmic reticulum and produce a lower-amplitude Ca^{2+} transient and twitch during subsequent cardiac cycles. Although we have found that concentrations of cocaine in the range of 10^{-4} M or higher may affect the interaction between aequorin and Ca^{2+} *in vitro*, such concentrations would not be expected to be achieved intracellularly in the present experiments. However, it must be noted that when applied in millimolar concentrations or higher, esteric local anaesthetics like cocaine may be expected to produce significant effects on sub-cellular Ca^{2+} handling (Blinks *et al.*, 1972). In the present study, effects of these large concentrations were not evaluated, not only because of a possible interaction with aequorin, but also because of the lack of clinical relevance of these very high concentrations.

Our data with skinned fibres also indicate that cocaine has the potential to alter the direct interaction of the cardiac contractile proteins with calcium, by decreasing both myofilament calcium sensitivity and maximal calcium-activated force (Figure 3). This effect must be due to a direct interaction of cocaine with the contractile proteins because these saponin-skinned fibres are devoid of functional autonomic nerve endings, the sarcolemma is removed as a diffusion barrier, and (in our preparations) the sarcoplasmic reticulum is destroyed. Free diffusion of cocaine into the sarcoplasm may explain why a 10^{-5} M concentration of cocaine produced a marked decrease in Ca^{2+} sensitivity in skinned cardiac muscle fibres, but not on intact papillary muscles stimulated with threshold voltage (see above). One implication of these results is that in a normally innervated heart, cocaine could decrease myofilament calcium responsiveness both by a drug-mediated rise in catecholamines and by its direct effects on the contractile apparatus. The mechanism of the direct myofilament action of cocaine remains to be determined, but could be related to an alteration in thin filament force regulation such as a change in the affinity of troponin-C for Ca^{2+} .

Toxic cardiovascular effects of cocaine

We and others have previously demonstrated that higher concentrations of drugs that increase intracellular cyclic AMP concentrations are often associated with the occurrence of Ca^{2+} oscillations and dysrhythmias (Orchard *et al.*, 1983; Wier *et al.*, 1983; Morgan & Morgan, 1984; Gwathmey & Morgan, 1985). Previous studies have shown that after-contractions and after-depolarizations occur with toxic doses

of most positive inotropic agents that increase intracellular $[\text{Ca}^{2+}]$, particularly those that also increase intracellular cyclic AMP concentrations (Morgan & Morgan, 1984). These so-called 'triggerable' responses are an important cause of dysrhythmogenesis in addition to the mechanisms of automaticity and re-entry. Experimental evidence suggests that these after-responses are produced by an oscillatory release of calcium from intracellular stores. This oscillatory Ca^{2+} release may be caused by increased sarcoplasmic levels of calcium produced by high doses of inotropic drugs, that is, calcium-induced calcium release. Of interest, despite the marked positive inotropic response that we observed in some experiments, which we believe to be due to the effects of endogenous noradrenaline, cardiac dysrhythmias were not observed in any of our experiments. This suggests that the arrhythmogenic effects of catecholamines may be offset by the Na^+ channel blocking properties of these concentrations of cocaine, at least in the ferret.

In summary, our findings suggest that an increase in intracellular calcium and increased isometric tension development are characteristic of low and moderate concentrations of cocaine in ferret working myocardium. However, they are not due to an increased myofilament Ca^{2+} responsiveness. Such an increase in contractility would be expected to translate into an increase in myocardial oxygen consumption, which would become significant in patients with coronary artery disease. Moreover, since these effects are mediated by increased cyclic AMP, we would also expect, in the intact animal or patient, a positive chronotropic effect to occur which would additionally increase myocardial oxygen demands. The negative inotropic effect which occurs at larger concentrations, appears to be due to Na^+ channel blocking effects on the sarcolemma and, perhaps, direct interaction with the contractile proteins, would only be clinically relevant for patients consuming very large amounts of cocaine. In some reported pathological series, however, cocaine concentrations in excess of 5×10^{-5} M have been found in the blood (Van Dyke *et al.*, 1976; Paly *et al.*, 1982).

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