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EFFECTS OF 8-(*N*,*N*-DIETHYLAMINO)OCTYL 3,4,5-TRIMETHOXYBENZOATE (TMB8) ON RAT ATRIAL MUSCLE

BASIL J. NORTHOVER
Department of Pharmacology, School of Pharmacy, Leicester Polytechnic, Leicester LE1 9BH, U.K.

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Abstract—Rat atria loaded *in vitro* with the dye INDO-1 produced fluorescence signals indicative of changes in cytoplasmic calcium ion concentration ($[Ca^{2+}]_c$). Such atria showed systolic/diastolic fluctuations in fluorescence indicative of a systolic rise and a diastolic fall in $[Ca^{2+}]$ while being superfused with a solution containing a normal Ca^{2+} concentration. Some atria were then exposed to a low $[Ca^{2+}]$ in the superfusate. This caused negative inotropism and fluorescence changes indicative of a decline in $[Ca^{2+}]_c$. Both of these responses were reversed by adding 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB8; 2 μM) to the superfusate. Some atria were exposed instead either to a low $[K^+]$ in the superfusate or to an ouabain-containing superfusate. These atria developed a contracture, associated with fluorescence changes indicative of a rise in $[Ca^{2+}]_c$. The addition of TMB8 (2 μM) now relaxed the contracture, and this was associated with fluorescence changes indicative of a decline in $[Ca^{2+}]_c$. Atria that were exposed for 15 min to a low $[Na^+]$ in the superfusate developed a period of positive inotropism, followed by a brief period of negative inotropism on return to the normal superfusate. The period of positive inotropism was associated with fluorescence changes indicative of a rise in $[Ca^{2+}]_c$ and the period of negative inotropism with a decline in $[Ca^{2+}]_c$ to below baseline levels. All of these responses were less marked in atria exposed throughout to superfusates containing TMB8 (2 μM). Some atria were loaded with the dye SNARF-1. This emits fluorescence signals indicative of changes in cytoplasmic pH (pH_c). These atria showed no systolic/diastolic fluctuation of fluorescence, but when superfused with a bicarbonate-free solution they displayed a change in fluorescence indicative of a decline in pH_c in response to the addition of either ouabain or TMB8. Similarities were found between the effects produced by TMB8 and those produced by amiloride or dichlorobenzyl amiloride, suggesting that all three agents inhibit plasmalemmal Na^+/Ca^{2+} and Na^+/H^+ exchange.

Pretreament of rat atria with 8-(*N*,*N*-diethylamino)octyl 3,4,5- trimethoxybenzoate (TMB8*) protects against some of the deleterious consequences of exposure *in vitro* to conditions of simulated ischaemia [1], but the mechanism of this protection is obscure. Protection occurs at concentrations as low as 2 μM, at which TMB8 exerts no inotropic effect during superfusion with a normal physiological salt solution [1]. TMB8 is known, however, to inhibit Ca release from intracellular stores [2–4], and this would explain the ability of high concentrations of TMB8 to reduce the contractile force of various muscles, but does not account for protection of atrial muscle at concentrations below the inotropic threshold. Other possible explanations, therefore, need to be considered, and several actions of TMB8 have been reported previously in certain tissues, although these were seen only at higher concentrations than 2 μM [5–8]. It seemed worthwhile, therefore, to explore possible interference by TMB8 with cation exchange processes across the plasmalemma, since these are known to contribute to ischaemic injury [9]. Na^+/Ca^{2+} and Na^+/H^+ exchanges were experimentally manipulated by various alterations of superfusate composition. The ability of TMB8 to modify the responses of atria to such manipulations was noted.

The effects of TMB8 in the present experiments were compared with those of known inhibitors of cation exchange. Amiloride inhibits Na^+/H^+ exchange, with a 50% inhibitory concentration in the range of 1–10 μM [10–12], and weakly inhibits Na^+/Ca^{2+} exchange, with a 50% inhibitory concentration in the range of 1–10 mM [10, 12]. Amiloride also blocks Na channels in certain epithelial cells at very low concentrations [13]. *N*-(3,4-Dichlorobenzyl) amiloride (DCBA), on the other hand, shows a 50% inhibitory concentration against both Na^+/Ca^{2+} and Na^+/H^+ exchange in the range of 10–100 μM [10–12, 14]. Intracellular protons that are produced during metabolism leave cells via at least two mechanisms [15]. Firstly, influxing Na^+ may exchange with effluxing H^+. Secondly, extracellular HCO_3^- may enter cells in exchange for effluxing Cl^-, or possibly in association with Na^+ entry via a co-transporter [16]. In either event, an entering HCO_3^- combines with an intracellular H^+ to produce carbonic acid, which then dissociates to yield CO_2 and H_2O, effectively removing a proton

* Abbreviations: TMB8, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate; DCBA, *N*-(3,4-dichlorobenzyl) amiloride; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate; *F*, fluorescence intensity of emission; Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulphonate; HS, Hepes superfusate; NS, normal superfusate; $[Na^+]_c$, $[K^+]_c$, $[Ca^{2+}]_c$, $[H^+]_c$, the cytoplasmic Na^+, K^+, Ca^{2+} and H^+ concentrations, respectively; $[Na^+]_s$, $[K^+]_s$, $[Ca^{2+}]_s$, $[H^+]_s$, the superfusate Na^+, K^+, Ca^{2+} and H^+ concentrations, respectively; pH_c, cytoplasmic pH.

Table 1. Composition of superfusates

Name of superfusate	Concentration of constituent (mM)						
	$CaCl_2$	NaCl	KCl	Sucrose	$NaHCO_3$	Hepes	Ouabain
Normal superfusate (NS)	2	138	4	0	10	0	0
Hepes superfusate (HS)	2	138	4	0	0	10	0
Low $[Ca^{2+}]_s$ superfusate	0.5	138	6	0	10	0	0
Low $[K^+]_s$ superfusate	1	138	1	0	10	0	0
Low $[Na^+]_s$ superfusate	2	69	4	138	10	0	0
Ouabain-containing NS	1	138	4	0	10	0	0.05
Ouabain-containing HS	1	138	4	0	0	10	0.05

from the cell. Some cells seem able to regulate intracellular pH (pH_c) via either process separately, with inhibition of one merely promoting the other. In the absence of extracellular HCO_3^-, however, or in the presence of an inhibitor of HCO_3^- entry such as 4- acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate (SITS), cells become more dependent upon Na^+/H^+ exchange for pH_c homeostasis. Inhibition of Na^+/H^+ exchange then causes a decline in pH_c [17]. A similar strategy was used in the present study to investigate inhibitors of Na^+/H^+ exchange.

MATERIALS AND METHODS

Atria were isolated from rat hearts and mounted on a plastic holder as described previously [18]. Atria were bathed, unless otherwise stated, with normal superfusate (NS) of the following composition (mM): NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 10, glucose 10, and gassed with 95% O_2 plus 5% CO_2 (Table 1). For some experiments the $NaHCO_3$ in the above fluid was replaced by an equal concentration of *N*-2-hydroxyethyl piperazine-*N*′-2-ethane-sulphonic acid which had been adjusted to pH 7.3 with NaOH. This is referred to as Hepes-superfusate (HS in Table 1), and was gassed with O_2 in place of carbogen. Atria were stimulated throughout an experiment at 4 Hz with 2 msec square pulses, each of 10 V. A thread was sutured to the left atrial appendage and connected to a force displacement transducer (type SB-IT, Nihon Khoden) at a diastolic tension of 100 mg. Changes in tension were recorded via a d.c. amplifier (type 5240) on a heated stylus recorder (Multitrace 2, Lectromed). Atria were allowed to equilibrate in NS for 1 hr before dye-loading or alteration to superfusate composition.

Dye-loading and fluorescence measurement. Atria were loaded with acetoxyethyl esters of either INDO-1 (from Sigma) or SNARF-1 (from Molecular Probes), A 50 μL aliquot of a stock solution of the appropriate dye ester in dimethyl sulphoxide was first mixed with 0.2 ml of a 0.3% solution in dimethyl sulphoxide of the non-ionic detergent Pluronic F-127 (a polyoxyethylene/polyoxypropylene co-polymer, supplied by Mazer Chemicals). This mixture was then shaken with 10 ml of superfusate (Table 1) to give a 5 μM dye ester solution, in which the atria were incubated for 1 hr at 34°. Ester solubility in aqueous media, and hence ease of ester penetration of the plasmalemma and subsequent hydrolysis to non-diffusible dye salts within the cytoplasm, are increased in the presence of Pluronic [18].

Dye-loaded atria, on the plastic holder, were positioned diagonally across a 1 cm square section quartz cuvette and maintained at 34° in a specially designed fluorimeter with one excitation monochromator and two emission monochromators, each with a photon-counting multiplier tube (the Alphascan System, Photon Technology International), as described in detail previously [18]. Epifluorescence emissions were collected digitally at 60 data points/sec from the front face and one side face of the cuvette. In the case of INDO-loaded atria excitation was at 360 nm, and the two emission monochromators were set at 400 and 500 nm respectively. The intensity of fluorescence (*F*) emitted at 400 nm by INDO-1 is increased by a rise of $[Ca^{2+}]$, but diminished at 500 nm. Changes in the ratio of emission intensities at these two wavelengths (*F*400/500) are a measure of changes in $[Ca^{2+}]_c$ [19]. In the case of SNARF-loaded atria fluorescence excitation was at 534 nm and the two emission monochromators were at 644 and 584 nm, respectively. The intensity of fluorescence emitted at 584 nm by SNARF-1 is increased by a rise in $[H^+]$, but diminished at 644 nm. Changes in the ratio of emission intensities at these two wavelengths (F644/584) are a measure of changes in pH_c between pH 6.5 and 7.5 [20]. Before dye-loading, however, the combined tissue and instrumentation autofluorescence was recorded and filed in computer memory, to be deducted later from signals generated by dye-loaded atria. Autofluorescence needs to be recorded for each experimental intervention, however, as it varies in different superfusates. All results presented in this report have been corrected in this way.

Manipulation of plasmalemma Na^+/Ca^{2+} *exchange.* This exchange operates bidirectionally and can be driven by an energy gradient provided by either cation [21]. Shattock and Bers [22] showed that in rat myocardium with a normal superfusate the exchange operates in a slightly Na^+ influxing (Ca^{2+} effluxing) mode during diastole. Three methods for manipulation of this exchange were used in the present experiments. Some atria were

transferred after equilibration in NS to a superfusate containing a very low $CaCl_2$ concentration, namely 0.5 mM, and a higher than normal KCl concentration, namely 6 mM, but otherwise with the same composition as NS. This is referred to as low $[Ca^{2+}]_s$ superfusate (Table 1), and was intended to lower $[Ca^{2+}]_c$ in part by reducing Ca entry via Ca channels during systole and in part by promoting Ca^{2+} efflux/Na^+ influx during diastole. An inhibitor of Na^+/Ca^{2+} exchange in this superfusate would be expected to increase $[Ca^{2+}]_c$ and tension values towards those seen in NS.

The second method used to manipulate Na^+/Ca^{2+} exchange was by reducing the superfusate NaCl concentration from 138 to 69 mM, while keeping isotonicity with added sucrose (Table 1). This was intended to load the myocytes with Ca by either retarding Ca efflux or possibly causing Ca influx through the Na^+/Ca^{2+} exchanger.

The third method used to manipulate Na^+/Ca^{2+} exchange was by inhibiting the plasmalemmal Na^+/K^+ pump, thereby elevating cytoplasmic Na^+ concentration ($[Na^+]_c$). This too would load the myocytes with Ca [23], either by retarding Ca efflux in exchange for Na influx, or possibly by reversing the direction of exchange.

Drugs. SITS (as its Na salt) and TMB8 (as its hydrochloride) were purchased (Sigma) and dissolved in water for use. Amiloride hydrochloride was purchased from Research Biochemicals Inc. DCBA was generously donated by William Henckler of Merck Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. Amiloride and DCBA were first dissolved in dimethyl sulphoxide and then diluted with superfusate for use. The final concentration of dimethyl sulphoxide in the superfusate was 0.1% or less.

Statistics. The results in this report are expressed as means ± SEM. A difference between two means was considered significant if the null hypothesis in a Student's *t*-test was rejected because $P < 0.05$.

RESULTS

Atria superfused with NS or HS

INDO-loaded atria exposed to NS for 1 hr showed systolic rises in both tension and *F*400/500 ratios with each electrical pacing stimulus, confirming earlier reports [18], but SNARF-loaded atria showed no significant systolic to diastolic fluctuation in *F*644/584 ratios (Table 2). Some atria were then transferred from NS to HS. After exposure to HS for a further 30 min there was no significant change in any of the measured parameters (Table 2).

Atria superfused with low $[Ca^{2+}]_s$ *superfusate*

Instead of transfer to HS, some atria were transferred from NS to low $[Ca^{2+}]_s$ superfusate. This caused a negative inotropic effect and a decline in systolic *F*400/500 ratios, which reached a nadir in both respects after about 10 min, remaining constant thereafter for at least 3 hr. The values presented in Table 2 were recorded after 30 min in this superfusate. Diastolic tension and diastolic *F*400/500 ratios did not change significantly after transfer to low $[Ca^{2+}]_s$ superfusate (Table 2) The *F*644/584 ratios likewise were unchanged by transfer of the atria from NS to low $[Ca^{2+}]_s$ superfusate (Table 2).

Atria with an inhibited Na^+/K^+ *pump*

Some atria were transferred from NS to low $[K^+]_s$ superfusate, which produced a positive inotropic response and a progressive rise in diastolic tension, with maximal tension being reached after about 45 min, after which time tension remained constant for 1–2 hr. The values listed in Tables 2–4 were obtained after 1 hr in this superfusate. Atria exposed to low $[K^+]_s$ superfusate showed enhanced systolic and diastolic *F*400/500 ratios compared with those obtained while in NS, but *F*644/584 ratios were not significantly different from those seen in NS (Table 2). Other atria were transferred from NS to NS containing 50 μM ouabain. This produced a pattern of tension and fluorescence changes (Table 2) similar to that produced by the low $[K^+]_s$ superfusate. When ouabain was added to HS rather than NS, however, atria showed only a small change in tension, but a significant decline in *F*644/584 ratios. Changes in systolic and diastolic *F*400/500 ratios were similar whether atria were exposed to ouabain in NS or HS (Table 2). Maximal tension and fluorescence effects were produced by exposing atria to ouabain for 40–60 min, but the values presented in Tables 2–4 were obtained after exposure to the drug for 1 hr.

Atria exposed to low $[Na^+]_s$ *superfusate*

Transfer of atria from NS to low $[Na^+]_s$ superfusate caused a positive inotropic response and a rise in diastolic tension, confirming earlier findings [23], but the partial contracture was poorly maintained (Fig. 1). After 15 min in low $[Na^+]_s$ superfusate, returning atria to NS again caused an early but brief period of negative inotropy (Fig.1), with developed tension falling below values seen previously while the atria were in NS. Tension responses and *F*400/500 ratios changed more or less in parallel with each other during these alterations in superfusate (Table 2), but no corresponding change in *F*644/584 ratios was seen.

Effects of TMB8

Atria in NS showed no significant change in peak systolic or end diastolic *F*400/500 ratios in response to addition of 2 μM TMB8, and neither was a significant inotropic effect observed (Table 2). At 20 μM, however, TMB8 caused a negative inotropic response that was maximal within 10 min, and associated with a decrease in peak systolic *F*400/500 ratios, although with no change in corresponding end-diastolic values (Table 2). At neither of these concentrations of TMB8 was there a significant change in the *F*644/584 ratio (Table 2).

In contrast to atria in NS, those in HS showed a negative inotropic response to 2 μM TMB8. This was maximally developed within 15 min and was associated with a decline in *F*644/584 ratios, although with no significant change in *F*400/500 ratios (Table 2). Atria superfused with NS containing 100 μM SITS responded mechanically to TMB8 in a similar way to atria in HS. Unfortunately, the fluorescence

Table 2. Effect of TMB8 on the atrial responses to various superfusates

Superfusate	TMB8 (μM)	Tension (g)		*F*400/500		*F*644/584
		Systolic	Diastolic	Systolic	Diastolic	
NS	0	0.32 ± 0.02*	0.09 ± 0.02*	1.14 ± 0.01*	1.02 ± 0.01*	1.14 ± 0.02
NS	20	0.17 ± 0.01†	0.08 ± 0.01†	1.09 ± 0.02†	1.02 ± 0.02	1.15 ± 0.03
NS	2	0.34 ± 0.02	0.09 ± 0.02	1.15 ± 0.02	1.03 ± 0.02	1.14 ± 0.03
HS	0	0.30 ± 0.02	0.10 ± 0.02	1.14 ± 0.01	1.02 ± 0.01	1.14 ± 0.02
HS	2	0.18 ± 0.03	0.06 ± 0.01	1.13 ± 0.02	1.02 ± 0.01	1.04 ± 0.03
Low [Ca]	0	0.16 ± 0.01†	0.09 ± 0.03	1.06 ± 0.01†	1.02 ± 0.02	1.14 ± 0.03
Low [Ca]	2	0.27 ± 0.03	0.10 ± 0.02	1.11 ± 0.02	1.02 ± 0.03	1.15 ± 0.04
Low [K]	0	0.46 ± 0.02†	0.25 ± 0.05†	1.21 ± 0.02†	1.13 ± 0.03†	1.15 ± 0.03
Low [K]	2	0.34 ± 0.03	0.13 ± 0.03	1.16 ± 0.02	1.06 ± 0.02	1.13 ± 0.02
Ouabain in NS	0	0.47 ± 0.03†	0.27 ± 0.04†	1.23 ± 0.02†	1.10 ± 0.01†	1.13 ± 0.02
Ouabain in NS	2	0.37 ± 0.03	0.16 ± 0.02	1.17 ± 0.01	1.06 ± 0.01	1.13 ± 0.03
Ouabain in HS	0	0.36 ± 0.04	0.12 ± 0.03	1.22 ± 0.02†	1.09 ± 0.02†	1.14 ± 0.04
Ouabain in HS	2	0.21 ± 0.03	0.09 ± 0.01	1.16 ± 0.01	1.04 ± 0.02	1.03 ± 0.03
Low [Na] (onset)	0	0.81 ± 0.05†	0.52 ± 0.04†	1.28 ± 0.05†	1.19 ± 0.05†	1.12 ± 0.04
Low [Na] (onset)	2	0.59 ± 0.04	0.33 ± 0.07	1.21 ± 0.04	1.12 ± 0.03	1.13 ± 0.05
Low [Na] (offset)	0	0.30 ± 0.04	0.17 ± 0.03†	1.15 ± 0.02	1.09 ± 0.02†	1.15 ± 0.05
Low [Na] (offset)	2	0.45 ± 0.04	0.22 ± 0.04	1.19 ± 0.03	1.08 ± 0.02	1.14 ± 0.03

Time of exposure to TMB8 was 15 min. Values marked † are significantly different from corresponding control values marked * . Onset values for low [Na] superfusate are the maximum values recorded during 15 min of exposure to that superfusate, whereas offset values are the minimum values recorded within the first 2 min after return to NS. There were between 6 and 12 observations for each recorded value. Pairs of corresponding values that are significantly different are bracketed together.

Table 3. Effect of amiloride on atrial tension responses to various superfusates

Superfusate	Amiloride (μM)	Tension (g)	
		Systolic	Diastolic
NS	0	0.29 ± 0.03*	0.10 ± 0.02*
NS	1000	0.29 ± 0.02	0.10 ± 0.03
NS + SITS	0	0.27 ± 0.02	0.09 ± 0.01
NS + SITS	40	0.20 ± 0.03	0.08 ± 0.01
HS	0	0.30 ± 0.02	0.09 ± 0.02
HS	40	0.22 ± 0.02	0.07 ± 0.01
Low [Ca]	0	0.15 ± 0.01†	0.08 ± 0.01
Low [Ca]	1000	0.24 ± 0.02	0.09 ± 0.03
Low [Ca]	40	0.16 ± 0.01	0.08 ± 0.02
Low [K]	0	0.46 ± 0.02†	0.25 ± 0.05†
Low [K]	40	0.36 ± 0.02	0.13 ± 0.04
Ouabain in NS	0	0.47 ± 0.03†	0.27 ± 0.04†
Ouabain in NS	40	0.34 ± 0.04	0.14 ± 0.04
Ouabain in HS	0	0.35 ± 0.04	0.12 ± 0.03
Ouabain in HS	40	0.26 ± 0.03	0.08 ± 0.02
Low [Na] (onset)	0	0.82 ± 0.04†	0.49 ± 0.06†
Low [Na] (onset)	1000	0.59 ± 0.05	0.31 ± 0.08
Low [Na] (onset)	40	0.80 ± 0.06	0.50 ± 0.05
Low [Na] (offset)	0	0.29 ± 0.06	0.18 ± 0.04†
Low [Na] (offset)	1000	0.45 ± 0.07	0.20 ± 0.04
Low [Na] (offset)	40	0.31 ± 0.04	0.21 ± 0.05

Time of exposure to amiloride was 15 min. Values marked † are significantly different from corresponding control values marked *. Onset values for low [Na] superfusate are the maximum values recorded during 15 min of exposure to that superfusate, whereas offset values are the minimum values recorded within the first 2 min after return to NS. There were between 6 and 12 observations for each recorded value. Pairs of corresponding values that are significantly different are bracketed together.

Table 4. Effect of DCBA on atrial responses to various superfusates

Superfusate	DCBA (μM)	Tension (g) Systolic	Diastolic
NS	0	0.29 ± 0.03*	0.10 ± 0.02*
NS	20	0.28 ± 0.02	0.09 ± 0.02
NS + SITS	0	0.27 ± 0.02	0.09 ± 0.01
NS + SITS	20	0.18 ± 0.03	0.07 ± 0.01
HS	0	0.30 ± 0.02	0.09 ± 0.02
HS	20	0.21 ± 0.03	0.07 ± 0.02
Low [Ca]	0	0.15 ± 0.01†	0.08 ± 0.01
Low [Ca]	20	0.25 ± 0.02	0.10 ± 0.02
Low [K]	0	0.46 ± 0.02†	0.25 ± 0.05†
Low [K]	20	0.31 ± 0.05	0.16 ± 0.05
Ouabain in NS	0	0.47 ± 0.03†	0.27 ± 0.04†
Ouabain in NS	20	0.33 ± 0.05	0.17 ± 0.04
Ouabain in HS	0	0.36 ± 0.04	0.12 ± 0.03
Ouabain in HS	20	0.21 ± 0.02	0.09 ± 0.02
Low [Na] (onset)	0	0.82 ± 0.04†	0.49 ± 0.06†
Low [Na] (onset)	20	0.63 ± 0.02	0.31 ± 0.06
Low [Na] (offset)	0	0.29 ± 0.06	0.18 ± 0.04†
Low [Na] (offset)	20	0.46 ± 0.05	0.18 ± 0.06

Time of exposure to DCBA was 15 min. Values marked † are significantly different from corresponding control values marked *. Onset values for low [Na] superfusate are the maximum values recorded during 15 min of exposure to that superfusate, whereas offset values are the minimum values recorded within the first 2 min after return to NS. There were between 6 and 12 observations for each recorded value. Pairs of corresponding values that are significantly different are bracketed together.

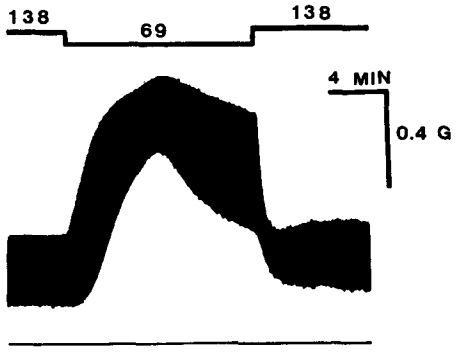

Fig. 1. Atrial tensions during exposure to NS, and then for the first 15 min after transfer to low $[Na^+]_s$ superfusate (NaCl concentration = 69 mM), followed by return to NS.

of SITS precluded an interpretation of optical signals from SITS-pretreated atria.

Atria exposed to low $[Ca^{2+}]_s$ superfusate for 15 min responded to addition of 2 μM TMB8 with a positive inotropic response and a rise in peak systolic *F*400/500 ratios, although *F*644/584 ratios did not change significantly (Table 2).

Atria that had been exposed to low $[K^+]_s$ superfusate, or to ouabain-containing NS, for 1 hr, responded to addition of 2 μM TMB8 with a decrease in both tension and *F*400/500 ratios, although with no significant change in *F*644/584 ratios (Table 2). These effects of TMB8 were maximally developed within 5–10 min.

Atria that had been pretreated with 2 μM TMB8 for 15 min, and then transferred to low $[Na^+]_s$ superfusate containing 2 μM TMB8 for a further 15 min, showed attenuated onset and offset responses compared with atria that had not been treated with TMB8 (Table 2).

Effects of amiloride and DCBA

Addition of 1 mM amiloride or 20 μM DCBA to atria in NS failed to cause a significant inotropic change, but both drugs caused negative inotropism in atria superfused either with HS or with NS containing 100 μM SITS (Tables 3 and 4). Both amiloride and DCBA fluoresced at wavelengths needed for studies using INDO-1 or SNARF-1, Moreover, since both drugs accumulate intracellularly [24] it was inappropriate to utilize fluorescence signals from atria treated with these two drugs to monitor changes in cation concentration.

As with TMB8, addition of 1 mM amiloride or 20 μM DCBA to atria that had been exposed to low $[Ca^{2+}]_s$ superfusate for 30 min caused a reversal of the negative inotropic effect of this superfusate, although amiloride at 40 μM was inactive in this respect (Tables 3 and 4).

Again as with TMB8, addition of 40 μM amiloride or 20 μM DCBA to atria that had been exposed to low $[K^+]_s$ superfusate, or to NS containing 50 μM ouabain for 1 hr, caused tension values to decline (Tables 3 and 4), and in the case of amiloride this confirms previous findings [14, 15, 25].

Atria that had been pretreated with 1 mM amiloride or 20 μM DCBA for 15 min before transfer from NS to low $[Na^+]_s$ superfusate, displayed an attenuated onset and offset tension response, although amiloride at 40 μM was ineffective (Tables 3 and 4).

In summary, therefore, the mechanical effects of TMB8, amiloride and DCBA were qualitatively similar to each other, as can be seen by comparing Tables 2–4.

DISCUSSION

INDO-1 has been used previously to monitor changes in $[Ca^{2+}]_c$ in rat atrial myocardium [18, 26], and found to be reliable. Although SNARF-1 has been used to monitor changes in pH_c in various tissues [27], its use in myocardium has not been reported previously. Both dyes have similar theoretical and practical limitations [27], including difficulties with calibration *in vivo*, only partial ion selectivity, leakage of dye from the cell (with or without photobleaching), some ionic buffering in the cytoplasm, and possible intracellular compartmentalization. Nevertheless, on present evidence, one may tentatively interpret changes in *F*400/500 and *F*644/584 ratios as indicative of changes occurring in $[Ca^{2+}]_c$ and pH_c, respectively.

A major determinant of the magnitude of systolic rises in $[Ca^{2+}]_c$, and thus of systolic tension development, is the amount of Ca available for release from the sarcoplasmic reticular store at the start of systole. In turn, this store is governed by the diastolic $[Ca^{2+}]_c$. This explains why changes in diastolic, as well as systolic, INDO-1 fluorescence in the present experiments correlated with the prevailing inotropic state.

Na^+/Ca^{2+} *exchange in low* $[Ca^{2+}]_s$ *superfusate*

As outlined in Materials and Methods, inhibitors of Na^+/Ca^{2+} exchange would be expected to exert a positive inotropic effect in this superfusate, and this was seen in the present experiments with amiloride, DCBA and TMB8. This is a new observation as far as TMB8 is concerned and suggests that all three drugs inhibit Na^+/Ca^{2+} exchange in rat atrial myocardium. Had these three drugs blocked Na or Ca channels, or inhibited Ca release from the sarcoplasmic reticulum, they would have potentiated and not overcome the negative inotropism seen in low $[Ca^{2+}]_s$ superfusate.

Na^+/Ca^{2+} *exchange in low* $[Na^+]_s$ *superfusate*

Responses of atria to this superfusate are divisible into those seen during the onset and the offset periods, respectively. Onset of exposure to low $[Na^+]_s$ superfusate would be expected to slow Na^+ influx/Ca^{2+} efflux, or perhaps reverse the exchanger to produce Na^+ efflux/Ca^{2+} influx. In either event, however, assuming that other contributors to net Ca flux are unchanged, myocytes would become loaded with Ca. An inhibitor of exchange, however, would be expected to cause inotropic effects of opposing signs, depending upon the prevailing direction of Na^+/Ca^{2+} exchange. If Ca^{2+} efflux still prevailed, albeit at a reduced rate, an inhibitor of exchange would be expected to reinforce the positive inotropism already created by this superfusate. In fact, amiloride, DCBA and TMB8 each opposed the positive inotropism caused by onset of exposure to low $[Na^+]_s$ superfusate, suggesting that Na^+/Ca^{2+} exchange was operating in Ca^{2+} influx mode at that stage.

Offset responses produced by return of atria from low $[Na^+]_s$ superfusate to NS would be expected to have increased Na^+ influx/Ca^{2+} efflux, on account of the low $[Na^+]_c$ that occurs at that stage in the myocardium [28]. Inhibitors of exchange would oppose the phase of temporary negative inotropism, therefore, on return of atria to NS. Such was the case in the present experiment with amiloride, DCBA and TMB8.

Any blockage of Na or Ca channels, or inhibition of Ca release from the sarcoplasmic reticulum would be expected to exert a negative inotropic effect during both the onset and the offset responses to low $[Na^+]_s$ superfusate. As mentioned above, however, an opposite effect to this was seen during periods of offset in the case of amiloride, DCBA and TMB8. This makes it seem unlikely that ion channel blockade was produced by these three drugs in rat atria, as was concluded also in connection with the experiments conducted in low $[Ca^{2+}]_s$ superfusate.

Na^+/Ca^{2+} *exchange in atria with an inhibited* Na^+/K^+ *pump*

Inhibiting the Na^+/K^+ pump would be expected to raise $[Na^+]_c$ and thereby also $[Ca^{2+}]_c$. The rise in $[Na^+]_c$ would either retard continuing Na^+ influx/Ca^{2+} efflux, or possibly reverse the direction of this cation exchange. If the exchange continued to operate in a Ca^{2+} efflux mode during diastole, albeit at a reduced rate, then inhibition of Na^+/Ca^{2+} exchange would be expected to exert a positive inotropic effect, whereas negative inotropism would be produced by such an inhibitor if the exchange operated in a Ca^{2+} influx mode. In fact, in the present experiments, amiloride, DCBA and TMB8 each exerted a negative inotropic effect on atria exposed to superfusates designed to inhibit the Na^+/K^+ pump. This suggests that in such superfusates there was indeed Ca^{2+} influx through the exchange. However, had amiloride, DCBA and TMB8 blocked Na or Ca channels, or inhibited Ca release from the sarcoplasmic reticulum, then this could have contributed to the observed negative inotropism. While atria were in low $[Ca^2]_{s+}$ and low $[Na^+]_s$ superfusates evidence was obtained that blockade of ion channels with these three drugs did not occur. It seems more likely, therefore, that the negative inotropic effect of these three drugs in atria with an inhibited Na^+/K^+ pump was also due to inhibition of the Na^+/Ca^{2+} exchanger, rather than an effect on ion channels.

Atrial Na^+/H^+ *exchange*

Amiloride at 40 μM is well known to inhibit Na^+/H^+ exchange [10–12]. TMB8 at 2 μM would seem from the present experiments also to inhibit this exchange. This is seen most clearly in the TMB8-induced negative inotropism in HS, which was associated with fluorescence changes indicative of a

decline in pH_c in this bicarbonate-free superfusate. In bicarbonate-containing NS, on the other hand, there was no negative inotropism with TMB8 at 2 μM. The decline in pH_c would be expected to have reduced the sensitivity of contractile proteins to the mechanical effects of the systolic rises in $[Ca^{2+}]_c$, and thus to explain the observed negative inotropism.

At concentrations in excess of 2 μM, TMB8 exerts a wide range of pharmacological actions, including blockade of Ca channels [29, 30] and interference with Ca release from the sarcoplasmic reticulum [2–4]. Both of these actions may have contributed to the observed ability of TMB8 at 20 μM in the present experiments to cause negative inotropism, together with fluorescence changes indicative of a decline in $[Ca^{2+}]_c$. At 2 μM, however, TMB8 showed no inotropic effect in NS, and no fluorescence change indicative of altered $[Ca^{2+}]_c$. Some individual effects of TMB8 at 2 μM in the present experiments, and particularly those in the presence of an inhibited Na^+/K^+ pump, are compatible with actions other than inhibited cation exchange. Nevertheless, there are two distinct sets of circumstances that produced results with TMB8 that are compatible only with an inhibited Na^{2+}/Ca^{2+} exchange, and this remains the simplest way of accounting for the overall pattern of observed effects with this drug at 2 μM. This constitutes a novel biochemical site of action for this compound. Although the evidence for this mode of action is presently only indirect it provides a possible explanation for the previously reported ability of this drug to protect rat atria against the deleterious effects of simulated ischaemia [1].

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