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COMPOUND EFFECTS OF BISULPHITE ION ON FROG SKELETAL MUSCLE

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Abstract—To characterize the structures of the nicotinic cholinoceptors in the frog rectus abdominis muscle, group-selective reagents have been used. Possible modifications of the excitation–contraction coupling have also been studied. The effect of the bisulphite ion on skeletal muscle has been demonstrated earlier at presynaptic and postsynaptic levels. Another report questioned whether these effects were significant to the contractile process in general. The present study shows that bisulphite induced qualitative and quantitative changes in the muscular response to acetylcholine and electrical stimulation. The intervention is likely at the level of excitation–contraction coupling as well as at the previously proposed sites.

Various reagents have been used to demonstrate the presence of a disulphide bond at cholinergic receptor sites [1–4]. Sodium bisulphite treatment of frog cutaneous pectoris muscle and electrophysiological examination indicated that disulphide bonds are functionally important at presynaptic and postsynaptic receptor sites: spontaneous presynaptic activity was increased, as was the sensitivity of the postsynaptic receptor to exogenous acetylcholine (ACh) in the presence of bisulphite [5, 6]. However, in a different study, bisulphite did not induce contractions in frog rectus abdominis muscle, nor was the treated and washed muscle more sensitive to ACh [7]. The authors concluded that either the structure of the nicotinic receptor in cutaneous pectoris was different from that in rectus abdominis, or that, more likely, the electrophysiological results were due to effects of bisulphite on surface fibers only.

We have examined the effect of sodium bisulphite on the contractile response of frog rectus abdominis muscle both to ACh and electrical stimulation. Our study clarifies part of the discrepancy in the earlier reports and reveals an additional site of action of bisulphite. It appears that electrically-evoked contractions of the muscle are also affected by bisulphite. However, responses to electrical stimulation are not blocked by gallamine. Consequently, the influence of bisulphite on such contractions is not mediated by nicotinic receptors. Bisulphite must have a third site of action in which the excitation–contraction process is more likely involved.

MATERIALS AND METHODS

Isolated rectus abdominis preparations of the frog *Rana pipiens* of either sex were suspended in an organ bath (60 ml) containing Ringer's solution of the following composition (mM): NaCl, 111.1; KCl, 1.9; $CaCl_2$, 1.1; $NaHCO_3$, 4.8; $NaH_2PO_4 \cdot 2H_2O$, 0.04; and glucose, 1.1. This was aerated and maintained at room temperature (20°). Each muscle was attached by means of either a cotton thread (for cholinergic stimulation) or a platinum electrode (for electrical stimulation) to an isotonic transducer. Contractions were recorded on a Yokogawa potentiometer (model 3046) under a constant tension of 1 g. Drugs used were sodium bisulphite (Baker), acetylcholine iodide (Eastman), and gallamine triethiodide (Sigma). Electrical stimulation was applied with a Grass stimulator (model S4K) using individual shocks of 50-msec duration. The voltages were selected to give the full range of mechanical responses to electrical stimulation. This varied from one muscle to the next, and fell between the limits of 5 and 14 V.

The experimental design was divided in five parts to clarify the following points:

(1) To evaluate the direct effect of sodium bisulphite on otherwise untreated preparations, five muscles were exposed to sodium bisulphite (1.6×10^{-6} to 16 mM) during 5 min without a preliminary control exposure to either ACh or electrical stimulation.

(2) To ascertain whether gallamine, at an appropriate concentration, induces a full blockade of the nicotinic receptors and that electrical stimulation is not affected by this substance, the effect of a 15-min treatment with gallamine (0.16 mM) on the contractile response of two series of five preparations to cholinergical and electrical stimulation was also studied.

(3) To investigate a possible cholinergical potentiation after treatment by different concentrations of sodium bisulphite, recordings were made of the response of another series of ten muscles to: (a) a 3-min exposure to ACh followed by washing and a 3-min rest, followed by (b) recordings of the response

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to a 5-min exposure to sodium bisulphite and, finally, by (c) recordings of the response to a 3-min exposure to ACh in the presence of the bisulphite.

(4) To establish a possible electrical potentiation following exposure to different doses of bisulphite, the entire protocol No. 3 was repeated using electrical stimulation in place of ACh stimulation, and observing the effect of different concentrations of sodium bisulphite.

(5) To examine the mechanism of action of sodium bisulphite at the end plate level, recordings of spontaneous miniature potentials were made from five preparations of the rectus abdominis muscle isolated from small specimens of *R. pipiens*. The muscles were pinned (inner side up) in a small chamber containing Ringer's solution and studied at room temperature (20–22°). Intracellular glass microelectrodes, filled with 3 M KCl, of 5–10 megohms resistance and low (< 5 mV) tip potential, were employed to penetrate the two top layers of muscle fibers. In most preparations, the connective tissue overlying the muscle fibers was thin enough to permit entry of the microelectrode tip, although in some cases penetration of the first fiber beneath the surface connective tissue was accompanied by apparent injury and depolarization, often with consequent discharge of action potentials. For this reason, readings from the second layer of fibers were considered to be more reliable. In two preparations, the connective tissue was thicker and had to be peeled off with five forceps to allow microelectrode entry.

Three preparations were used to record spontaneous miniature potentials in normal Ringer's solution and in Ringer's solution containing 1.6×10^{-3} M sodium bisulphite. Two preparations were used to record spontaneous miniature potentials sequentially from a few fibers in normal Ringer's, 1.6×10^{-6} M ACh, 1.6×10^{-3} M sodium bisulphite, and in 1.6×10^{-3} M sodium bisulphite and 1.6×10^{-6} M ACh (as in the protocol given above for mechanical recordings, but with 6-min exposure times to the drugs to allow more time to record from impaled fibers). In each fiber impaled, spontaneous miniature potentials were counted over 1–2 min to obtain a measure of their frequency. Data were then pooled to provide an overall picture of the results.

RESULTS

Sodium bisulphite (1.6×10^{-6} to 16 mM) had no direct contractile effect on muscles that had not been given a prior exposure to either ACh or electrical stimulation.

Gallamine (0.16 mM) reversibly blocked the effects of ACh (3-min exposures to 1.6×10^{-3} to 1.6×10^{-2} mM) on muscle preparations. It also blocked the effects of sodium bisulphite (5-min exposures to 16 mM) but did not modify the response of muscles to electrical stimulation (stimuli varying from 5 to 14 V, 50-msec duration).

Following a control dose of ACh (1.6×10^{-3} to 1.6×10^{-2} mM), sodium bisulphite, up to 1.6 mM, had no contractile effect but increased the response to ACh (Table 1). The cholinergical potentiation was porportional to the concentration of sodium bisulphite (Fig. 1) and required a pretreatment of

Table 1. Effects of sodium bisulphite on the contractile response of frog rectus muscle to ACh*

ACh (mM)	Contraction (mm)	$NaHSO_3$ (mM)	Contraction (mm)	Combined $NaHSO_3$: ACh (mM) (mM)	Contraction (mm)	Potentiation of ACh (%)	n
1.6×10^{-3}	3.45 ± 0.64	1.6	0	$1.6 : 1.6 \times 10^{-3}$	4.75 ± 0.67	52.56 ± 12.22	10 (10)
1.6×10^{-2}	9.64 ± 0.67	1.6	0	$1.6 : 1.6 \times 10^{-2}$	10.06 ± 0.42	11.86 ± 4.78	10 (6)
1.6×10^{-3}	3.82 ± 0.62	16	3.27 ± 0.61	$16 : 1.6 \times 10^{-3}$	8.6 ± 0.61	119.88 ± 47.13	10 (7)
1.6×10^{-2}	8.84 ± 0.52	16	3.77 ± 0.57	$16 : 1.6 \times 10^{-2}$	11.4 ± 0.83	27.07 ± 5.60	10 (3)

* The two sets of experiments using 1.6 mM $NaHSO_3$ were carried out on the same series of ten muscles. The potentiation was observed in six cases out of ten during a second treatment by $NaHSO_3$, and subsequent exposures to 1.6 mM $NaHSO_3$ decreased substantially the rate of the cholinergic potentiation resulting finally in an abolition of this phenomenon. With 16 mM $NaHSO_3$, experiments could not be repeated with the same muscles due to the irreversible nature of the potentiated response; each set of experiments was therefore done on a different series of ten muscles. When the contractile response due to $NaHSO_3$ was high, the cholinergic potentiation could not be observed: the number of muscles out of ten showing potentiation is indicated as (*n*). Results are expressed as mean ± S.E.M. Percent potentiation values represent, in each case, averages for the muscles showing potentiation. The possibility of pH artifacts was eliminated after studying acetic acid controls.

Table 2. Effects of sodium bisulphite on the contractile response of frog rectus muscle to electrical stimulation*

Stimulus (V)	Contraction (mm)	$NaHSO_3$ (mM)	Contraction (mm)	Combined $NaHSO_3$: Stimulus (mM) (V)	Contraction (mm)	Potentiation of the electrical stimulus (%)	n
5–12	2.36 ± 0.03	1.6	0	1.6 : 5–12	3.33 ± 0.41	41.94 ± 5.91	10 (10)
7–14	8.13 ± 0.63	1.6	0	1.6 : 7–14	8.31 ± 0.16	4.05 ± 0.85	10 (5)
5–9	2.98 ± 0.54	16	1.11 ± 0.18	16 : 5–9	5.19 ± 0.55	56.14 ± 15.25	10 (10)
5–13	8.27 ± 0.42	16	2.08 ± 0.62	16 : 5–13	10.35 ± 1.11	22.26 ± 6.16	10 (6)

* The intensity of the electrical stimulus was selected in order to obtain low and high level electrical responses, and the range of stimuli reflects a greater biological variation toward this type of stimulus than toward the cholinergic one. The two sets of experiments using 1.6 mM $NaHSO_3$ were carried out on the same series of ten muscles, a second treatment by $NaHSO_3$ resulting in the observation of the electrical potentiation in only five cases. This was not possible with 16 mM $NaHSO_3$ due to its fatiguing effect on the muscle; consequently different sets of ten muscles were used. A higher contractile response to $NaHSO_3$ also eliminated the possibility of observing the potentiation phenomenon. The number of muscles out of ten showing potentiation is represented by (n). Results are expressed as mean ± S.E.M. Percent potentiation values represent, in each case, averages for the muscles showing potentiation. Possible pH artifacts were excluded by examining acetic acid controls.

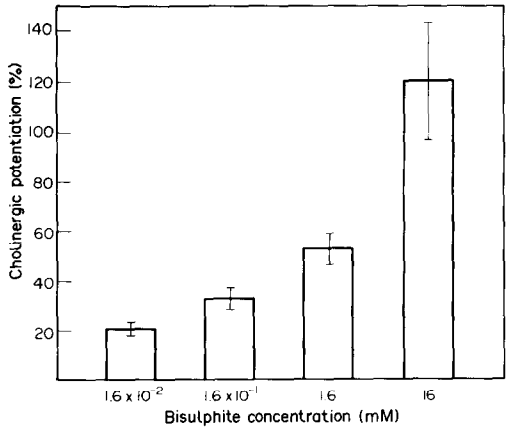

Fig. 1. Effects of sodium bisulphite on cholinergic responses of frog rectus muscles. ACh was used at a dose of 1.6×10^{-3} mM. Each concentration of bisulphite was tested on a different series of ten muscles. Values represent means ± S.E.M. as a percentage of cholinergic potentiation.

the preparation with ACh and the concomitant presence of bisulphite during the second application of ACh. The higher (16 mM) concentration of bisulphite elicited, by itself, muscular contractions which were reversibly blocked by gallamine (0.16 mM). The response to ACh was also potentiated by bisulphite at 16 mM. There was also a qualitative change, since the muscle failed to relax upon repeated washing with Ringer's solution. This phenomenon precluded the examination of complete dose–response curves in individual muscles.

Strikingly parallel results were obtained when muscles were stimulated electrically. After a control stimulus (5–14 V), bisulphite alone had no agonist effect up to 1.6 mM, but at this concentration poten-tiated the response to a second electrical stimulus of identical strength and duration, applied in the presence of the bisulphite (Table 2). The electrical potentiation increased with the concentration of sodium bisulphite (Fig. 2) and needed a pretreatment of the preparation with an electrical stimulation and the concomitant presence of bisul-

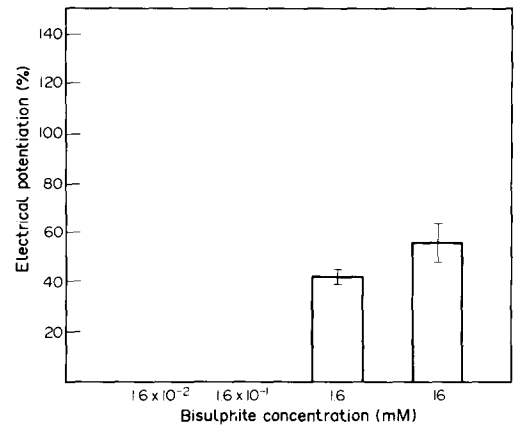

Fig. 2. Effects of sodium bisulphite on electrical responses of frog rectus muscles. Each concentration of bisulphite was tested on a different series of ten muscles. Values represent means ± S.E.M. as a percentage of electrical potentiation.

phite. Following an initial electrical stimulus, the higher concentration of the bisulphite (16 mM) induced muscular contractions, and potentiated contractions caused by a second, identical electrical stimulus. When the response to the bisulphite itself was pronounced, the potentiated response to the second electrical stimulus was irreversible. Neither termination of the electrical stimulation nor repeated washing with Ringer's solution relaxed the muscle.

Since bisulphite has a pronounced effect on spontaneous release of transmitter at the neuromuscular junction of the frog cutaneous pectoris muscle [6], tests were made on preparations of the rectus abdominis muscle to determine whether the potentiating effects of bisulphite could reasonably be attributed to a very large increase in spontaneous transmitter release, leading to maintained depolarization or action potentials.

Penetrations of muscle fibers in the top two layers of the inner (dorsal) surface in the intermediate segments of the muscle showed resting potentials ranging from 75 to 95 mV. Penetrations made through the connective tissue sheath often resulted in spontaneous action potentials, hence some of the fibers recorded from were damaged during penetration. Of the fibers penetrated, approximately 60% showed spontaneous miniature potentials of 0.5 to 1.5 mV amplitude. These usually occurred at a low rate; the highest frequency observed was 20/min, and the lowest 0.5/min. The mean frequency for 20 fibers was 3.7/min (± 0.28 S.E.M.).

Addition of sodium bisulphite did not result in a large increase in rate of discharge. In ten fibers showing spontaneous miniature potentials, the mean frequency was 6.4/min (± 0.64 S.E.M.).

In two preparations (one with sheath intact, and one with the sheath partly removed), spontaneous potentials were observed after exposure to ACh. Only two fibers showing spontaneous miniature potentials were sampled in each preparations after each change in solution due to time constraints. This was enough to establish that large increases in spontaneous potentials did not occur, and that ongoing spontaneous action potentials did not appear with the treatments employed.

Mean values for spontaneous miniature potentials from the pooled results were: in 10^{-6} M ACh, 2.4/min; in 1.6×10^{-3} M sodium bisulphite following a wash in Ringer's solution, 4/min; in sodium bisulphite (1.6×10^{-3} M) and ACh (1.6×10^{-6} M), 6.5/min. Two fibers showed isolated short-lasting bursts of spontaneous miniature potentials in the latter solution.

DISCUSSION

The present data show that the bisulphite ion interacts with both cholinergic and electrical stimuli to potentiate muscular contraction. The potentiation required that a prior stimulus of the corresponding type be applied. It was also necessary that the bisulphite be present at a minimum concentration of 1.6 mM during the application of the second stimulus. An earlier study [7] failed to demonstrate this potentiation because the bisulphite was washed out prior to application of ACh.

The sensitization produced is not due to elevation of the frequency or the magnitude of spontaneous miniature potentials, which we have shown are not dramatically affected by bisulphite in the frog rectus abdominis muscle. The low frequency of the spontaneous miniature potentials in this preparation, and their relative lack of response to bisulphite, are in contrast to the results obtained in the cutaneous pectoris muscle [6]. Previous electrophysiological work on the rectus abdominis muscle in another species, *Rana temporaria*, had indicated that the majority of fibers in this muscle are of the "twitch" type, with about 10% "tonus" type on the ventral surface [8]. Thus, we were probably recording mostly from "twitch" fibers, as evidenced also by the relatively high resting membrane potentials and the occurrence of spontaneous action potentials upon injury. The reason for the low frequency of spontaneous miniature potentials is not evident at present.

An alternative mechanism of sensitization, implicating excitation–contraction coupling, is supported by our results using electrical stimulation. Since gallamine failed to block responses to this stimulus, although it did block ACh-induced contractions, the responses were independent of the effect of ACh, from any source, on postsynaptic receptors.

Recent studies using other membrane-active anions, in particular perchlorate [9, 10], have indicated that excitation–contraction coupling can be markedly affected, possibly by an action on the gating mechanisms which are activated in the transverse tubular system by membrane depolarization and cause Ca^{2+} release from the sarcoplasmic reticulum. Bisulphite may also affect the gating mechanism of the transverse tubular system. Further more detailed work on single muscle fibers is required to test this hypothesis.

We suggest that an initial depolarization–repolarization cycle facilitates either accessibility or susceptibility of an excitation–contraction coupling site to the bisulphite ion. Sensitization of this site by the ion increases subsequent nicotinic responses to ACh or to the bisulphite ion itself, as well as increasing the responses to electrical stimuli. High concentrations of bisulphite cause irreversible changes in the sensitized site, in which case further depolarizations, due to ACh or to electrical stimulation, become permanent.

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