Br. J. Pharmac. (1980), **69**, 207–214

ADVERSE EFFECTS OF ARTIFICIAL BUFFERS ON CONTRACTILE RESPONSES OF ARTERIAL AND VENOUS SMOOTH MUSCLE

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1 *In vitro* studies were undertaken on rat aortic strips and portal vein segments in order to determine whether or not several commonly used artificial buffers, i.e., tris(hydroxymethyl) aminomethane (Tris), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), morpholine propanesulphonic acid (MOPS), N,N bis(2-hydroxyethyl) glycine (BICINE) and 1,4-piperazinediethanesulphonic acid (PIPES), can exert direct actions on vascular smooth muscle.

2 All artificial buffers used in 5 mM concentrations were found to inhibit development of spontaneous mechanical activity.

3 Tris, HEPES, MOPS, BICINE and PIPES markedly attenuated contractions induced by adrenaline, angiotensin and KCl. The fast phase components of the agonist-induced contractions were either obliterated or reduced in the presence of the artificial buffers. The sustained slow phase components were greatly reduced and retarded by the artificial buffers.

4 The relative order of artificial buffer potency (i.e., from 100% to 14% inhibition) seems to depend upon the agonist and type of smooth muscle.

5 All of these inhibitory effects were reversible, since normal contractile responses and spontaneous mechanical activity could be obtained by simply reincubating the smooth muscles in Krebs-Ringer bicarbonate buffer.

6 A variety of pharmacological antagonists failed to mimic or affect the inhibitory effects of Tris, HEPES, MOPS, PIPES and BICINE.

7 These data show that five of the most commonly used artificial buffers, to study muscles *in vitro*, exert adverse effects on contractility of arterial and venous smooth muscle.

Introduction

Artificial amines and synthetic organic substances such as tris(hydroxymethyl) aminomethane (Tris), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), morpholine propanesulphonic acid (MOPS), N,N bis(2-hydroxyethyl) glycine (BICINE) and 1,4-piperazinediethanesulphonic acid (PIPES), are commonly used as buffering agents in physiological media for studies of the role of Ca^{2+} in excitation-contraction coupling events in muscle. These artificial buffers have also been widely used for the study of calcium fluxes, calcium binding and in the calculation of distribution of this cation in various tissues; they have also been substituted for phosphate and HCO_3^- in order to prevent precipitation of various di- and trivalent cations, which are helpful in delineating the transmembrane fluxes of calcium and its binding sites.

We have recently found that Tris buffer inhibits Ca^{2+}- and drug-induced contractile responses, as well as exchangeability and uptake of radiocalcium in smooth muscle of rat aorta and portal vein (Turlapaty, Altura & Altura, 1978; 1979a). In view of these recent studies, and other reports which describe inhibitory effects of Tris on responses in several other types of excitable membranes (Lorkovic, 1972; Dresel, 1974; Davidoff & Sears, 1975; Gillespie & McKnight, 1976; Wilson, Clark & Pellmar, 1977), there was a need to determine whether the newer types of synthetic hydrogen ion buffers covering the range of pKa = 6.15 to 8.35 (Good, Winget, Winter, Connolly, Izawa & Singh, 1966) can be safely used to study contractility of smooth muscles. The studies presented herein indicate that 5 mM concentrations of HEPES, MOPS, BICINE, PIPES, as well as Tris, markedly inhibit spontaneous mechanical activity and agonist-induced contractile responses in two different types of smooth muscle when compared to results obtained in a phosphate- and bicarbonate-buffered medium.

0007-1188/80/060207-08 $01.00

Methods

Animals, smooth muscle preparations and experimental protocol

Aortic strips and portal veins, obtained from male Wistar rats (300 to 400 g) after decapitation, were set up isometrically *in vitro* as described previously (Altura & Altura, 1975) and equilibrated for 2 h in normal Krebs-Ringer bicarbonate solution (Table 1). The tissues were aerated with a 95% O_2: 5% CO_2 mixture and kept at 37°C at a pH of 7.40. The vascular strips were attached to force transducers (Grass FT .03c) for isometric recording with recording equipment identical to that described previously (Altura & Altura, 1970). The incubation media were routinely changed every 10 to 15 min as a precaution against interfering metabolites (Altura & Altura, 1970). After the 2 h incubation period in normal Krebs-Ringer bicarbonate, the tissues were stimulated to contract with single doses (ED_{50} to ED_{60}) of adrenaline, angiotensin or potassium chloride. Subsequently, after relaxation in normal Krebs-Ringer bicarbonate, the tissues were exposed in random, sequential fashion for 60 min periods each, to modified-Ringer solutions containing one of the five artificial buffers: Tris, HEPES, MOPS, PIPES or BICINE (see Table 1 for composition of salt solutions). Each of these solutions, containing an artificial buffer, was aerated with 100% oxygen. All salt solutions were kept isosmolar and maintained at a pH of 7.40 (see Table 1). After incubation for 60 min in each artificial buffer, the tissues were re-stimulated with the agonists. At various times during, and at the conclusion of, each experiment, the tissues were reincubated in normal Krebs-Ringer bicarbonate to determine if the smooth muscle preparations could still respond normally to the various agonists. Only one type of agonist was used on any one vascular preparation. The results of these experiments are expressed in mg of developed isometric tension. In other experiments, aortic strips ($n = 6$) and portal veins ($n = 6$) were exposed separately to various specific pharmacological antagonists for 10 min or to a prostaglandin synthetase inhibitor (indomethacin, 1.0 μg/ml) for 20 min. After the preincubation periods the aortae were stimulated with a submaximal dose of KCl ($\cong ED_{50}$). Both KCl-contracted aortae and spontaneous contracting portal veins were then exposed to the artificial buffers (5 mM concentrations) in order to observe whether: (a) the inhibitory actions of Tris, HEPES, MOPS, PIPES and BICINE would be affected by α-adrenoceptor blockade (phentolamine, 0.1 μg/ml), β-adrenoceptor blockade (propranolol hydrochloride 0.5 μg/ml), 5-hydroxytryptamine (5-HT) receptor blockade (methysergide, 0.5 μg/ml), or histamine receptor blockade (diphenhydramine hydrochloride 0.5 μg/ml); and (b) prostaglandins could have any intermediate role in the inhibitory responses observed with the artificial buffers. All concentrations of these drugs described above produce specific antagonism to their respective agonists on rat aorta and portal veins studied *in vitro* (Altura & Altura, 1974; 1975; Turlapaty, Altura & Altura, 1979b).

Statistical analyses

Where appropriate, the data are expressed as means ± s.e. Statistical analyses were performed by means of Student's t test and considered significant if $P < 0.05$. All t values obtained for the artificial buffers in this study are significantly different from those obtained for the bicarbonate controls.

Buffers, agonists and antagonists

The following buffers were used: tris (hydroxymethyl) aminomethane (Nutritional Biochemicals), 1,4-piperazinediethanesulphonic acid (PIPES, Boehringer Mannheim Biochemicals), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, Boehringer Mannheim Biochemicals), 4-morpholine propanesulphonic acid (MOPS, Boehringer Mannheim Biochemicals), N,N, bis (2-hydroxyethyl) glycine (BICINE, Research Organics, Inc.), $NaHCO_3$ (Fisher Scientific, A.C.S. certified), and KH_2PO_4 (Fisher Scientific, A.C.S. certified). The pharmacological agonists used were: adrenaline (Adrenalin chloride, Parke-Davis), angiotensin II amide (Hypertensin, Ciba-Geigy), and KCl (Fisher Scientific, A.C.S. certified). The pharmacological antagonists used were: phentolamine (Regitine, Ciba-Geigy Co.), methysergide (Sandoz Pharmaceuticals), atropine sulphate (Mann Research Laboratories), propranolol hydrochloride (Aldrich Chemical Co.), indomethacin (gift from Merck Sharp and Dohme), and diphenhydramine hydrochloride (Benadryl, Parke Davis and Co.). The concentration of each buffer is expressed as mM.

Results

Influence of artificial buffers on development of spontaneous mechanical activity

Figure 1 shows recordings of typical changes in spontaneous mechanical activity in four portal veins incubated in Krebs-Ringer bicarbonate and 5 mM concentrations of MOPS, HEPES, BICINE and PIPES-Ringer solutions. All of the artificial buffers attenuated the magnitudes of the mechanical spikes (i.e., tensions developed), and initially (during first 5 to 10 min of incubation) increased the frequency of por-

Table 1 Composition of salt solutions (mM)

Salt solution	NaCl	KCl	$CaCl_2$	KH_2PO_4	$MgSO_4$	$NaHCO_3$	Tris	HEPES	MOPS	PIPES	BICINE	NaOH	HCl	Glucose	pH
Bicarbonate-Ringer	118.0	4.7	2.5	1.2	1.2	25	—	—	—	—	—	—	—	10	7.40
Tris-Ringer	134.8	5.9	2.5	—	1.2	—	5.0	—	—	—	—	—	5.0	10	7.40
HEPES-Ringer	135.5	5.9	2.5	—	1.2	—	—	5.0	—	—	—	3.5	—	10	7.40
MOPS-Ringer	135.5	5.9	2.5	—	1.2	—	—	—	5.0	—	—	3.5	—	10	7.40
PIPES-Ringer	128.0	5.9	2.5	—	1.2	—	—	—	—	5.0	—	11.0	—	10	7.40
BICINE-Ringer	138.0	5.9	2.5	—	1.2	—	—	—	—	—	5.0	1.2	—	10	7.40

Each solution has a calculated osmolarity = 317.7 mosmol/l.

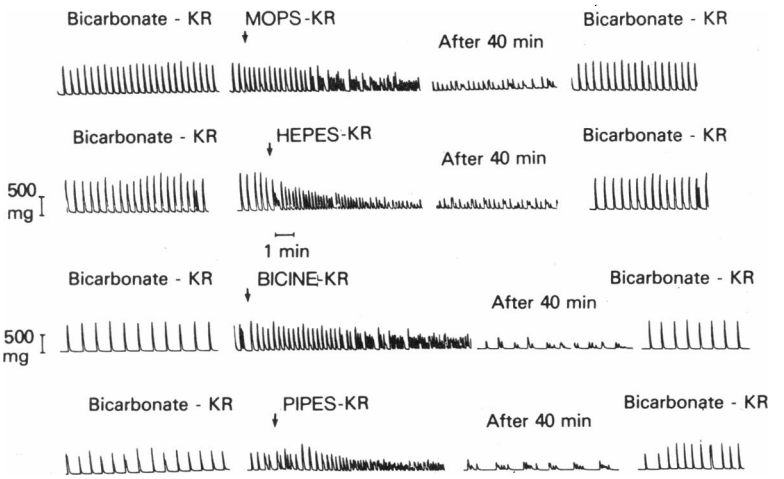

Figure 1 Influence of MOPS, HEPES, BICINE and PIPES buffers (5 mM) on spontaneous mechanical activity in rat portal veins. Each panel represents the successive effects of incubation in normal Krebs-Ringer bicarbonate, artificial buffered-Ringer solutions, and back into normal Krebs-Ringer bicarbonate. Subsequent changes of media are indicated by arrows. Bars on left represent tension in mg.

tal vein spontaneous mechanical activity. However, as the time of incubation approached 15 to 20 min, the artificial buffers resulted in significant decreases in frequency of the spontaneous contractions, and often by 40 min the frequencies exhibited a burst-like pattern with periods of quiescence lasting 1 to 2 min between the bursts of spontaneous contractions (Figure 1). When the artificial buffers were washed out and the tissues reincubated in normal Krebs-Ringer bicarbonate, the vascular smooth muscles exhibited full recovery of spontaneous mechanical activity within 30 min. The effects of the artificial buffers were similar in tissues initially incubated in either normal Krebs-Ringer bicarbonate or the artificial buffers. Either way, the effects of the five different artificial buffers were reversible.

Aortae incubated in normal Krebs-Ringer bicarbonate exhibited significant degrees of spontaneous mechanical activity at various times throughout the experiment, similar to that described previously (Altura & Altura, 1974). None of the aortae, however, exhibited any spontaneous mechanical activity when incubated in any of the five different artificial buffers.

Influence of artificial buffers on adrenaline-, angiotensin II- and potassium chloride-induced contractions of rat aorta and portal vein

In normal Krebs-Ringer bicarbonate, adrenaline, angiotensin and potassium chloride-induced contractions of aortae consisted of distinct fast and slow components. However, when the same aortae were incubated in one of the artificial buffers (5 mM) for 30 min and then exposed to the agonists, the fast components either disappeared (e.g., angiotensin, KCl) or were greatly slowed (e.g., adrenaline) (Figure 2). It is noteworthy that the slow components, unlike those seen in normal Krebs-Ringer bicarbonate, except for BICINE, were usually not sustained in the artificial buffers (Figure 2). In addition, the magnitudes of the agonist-induced contractions were considerably reduced in the artificial buffers (from 100% inhibition in the case of tissues incubated in Tris and stimulated with KCl to 14% inhibition in the case of BICINE incubation and stimulation with adrenaline) (Figure 2, Table 2). Also, it is important to note that the relative order of inhibitory potency of the artificial buffers on aortic smooth muscle appears to depend upon the agonist and buffer (Table 2). When the artificial buffer media were replaced with normal Krebs-Ringer bicarbonate, the agonist-induced contractile responses regained their full fast and slow components, as well as their potency (Figure 2). Similarly, all five artificial buffers greatly attenuated contractions induced by adrenaline, angiotensin and potassium chloride in rat portal venous smooth muscle (Figure 2). Here too, the relative order of inhibitory potency of the five buffers depended upon the agonist (Table 3). Interestingly, all five artificial buffers exhibited a similar degree of inhibition on angiotensin-induced contractions in the portal vein (Table 3). The inhibitory effects of all five artificial buffets were completely reversed when the portal veins were reincubated for 30 min in Krebs-Ringer bicarbonate solution (Figure 2).

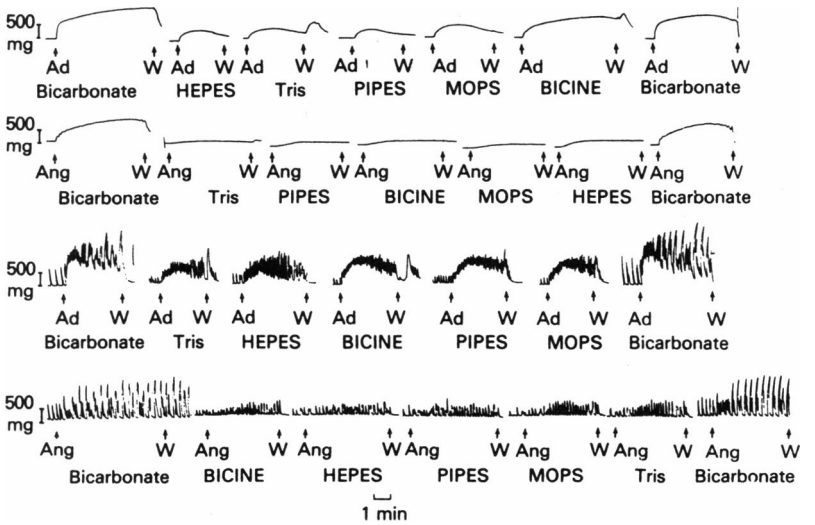

Figure 2 Effects of HEPES, Tris, PIPES, MOPS and BICINE buffers (5 mM) on adrenaline (Ad)- and angiotensin II (Ang)-induced contractions of two rat aortae (upper two panels) and portal veins (lower two panels). The concentrations of adrenaline and angiotensin II used for the aortae and portal veins were, respectively: aortae (1.15×10^{-8} M; 9.7×10^{-9} M), portal veins (9.7×10^{-8} M; 4.9×10^{-9} M). Bars on left represent tension in mg.

Table 2 Effects of buffers on adrenaline, angiotensin-, and potassium-induced contractile responses in rat aortae

Buffer	n	*Isometric developed tension* (mg)	% *inhibition*	t *value*
Adrenaline				
Bicarbonate	24	1106.0 ± 58.0		
HEPES	10	285.0 ± 50.0	74%	10.72
Tris	6	325.0 ± 36.0	71	11.44
PIPES	4	337.5 ± 51.5	69	9.91
MOPS	8	543.5 ± 88.00**	51	5.33
BICINE	6	950.0 ± 36.5***	14	2.27
Angiotensin				
Bicarbonate	11	913.5 ± 74.0		
Tris	4	87.5 ± 23.5	90%	10.63
PIPES	4	100.0 ± 35.0	89	9.94
BICINE	4	125.0 ± 32.0	86	9.78
MOPS	4	150.0 ± 20.0	84	9.96
HEPES	4	212.5 ± 74.5	77	9.47
Potassium				
Bicarbonate	15	836.5 ± 42.0		
Tris	6	0	100%	19.91
MOPS	4	162.5 ± 68.5*	81	8.39
BICINE	4	162.5 ± 117.5	81	5.40
HEPES	10	285.0 ± 95.0*	66%	5.31
PIPES	4	400.0 ± 192.5	52	2.21

Equipotent doses of agonists were used: adrenaline (1.15×10^{-8} M), angiotensin (9.7×10^{-9} M) potassium (1×10^{-2} M). Values are means ± s.e. mean. n = number of animals. All buffers were used in 5 mM concentration except bicarbonate 25 mM. t value represents difference from bicarbonate controls. Significantly different from * Tris, ** HEPES and *** PIPES

Failure of pharmacological antagonists and of a prostaglandin synthetase inhibitor to mimic or affect artificial buffer inhibition

Use of phentolamine, propranolol, methysergide, atropine, and diphenhydramine in concentrations which specifically antagonized ED_{50} responses of their respective agonists, did not change the spontaneous mechanical activity of the portal veins nor prevent (or modify) the inhibitory effects of Tris, HEPES, MOPS, BICINE and PIPES on either portal veins or KCl-contracted aortae. Although indomethacin attenuated the portal vein spontaneous contractions 30 to 50%, it did not alter the inhibitory effects of the artificial buffers either in the veins or in the aortae ($n = 6$ animals).

Discussion

The results of this study clearly demonstrated that five commonly used artificial buffers, Tris, HEPES, MOPS, BICINE and PIPES, exert depressant effects on spontaneous mechanical activity and agonist-induced contractile responses of rat aortic and venous smooth muscle. At first glance, one might be tempted to explain the adverse effects of these artificial buffers as being solely a reflection of the absence of HCO_3^- and/or PO_4^{-2} anions. Recent experiments from our laboratory indicate that: (1) as the concentration of HCO_3^- ions are progressively reduced from 25 mM to 5 mM (pH maintained at 7.4 by adding NaOH) in the bathing media, the contractile responses induced by adrenaline, angiotensin and KCl in both the rat aorta and portal vein are not significantly altered (Turlapaty *et al.*, 1979b); (2) removal of the 1.2 mM PO_4^{-2} from the Krebs-Ringer bicarbonate solutions does not alter the agonist-induced contractions (unpublished findings); and (3) the relative order of inhibitory potency of the artificial buffers is different for each agonist.

Since the inhibitory effects of the artificial buffers can be totally reversed by simply re-incubating the smooth muscle preparations in normal Krebs-Ringer bicarbonate for short periods of time (e.g., 30 to 45 min), this would argue against a possible cytotoxic

Table 3 Effects of buffers on adrenaline-, angiotensin-, and potassium-induced contractile responses in rat portal veins

Buffer	n	*Isometric developed tension* (mg)	% *inhibition*	t *value*
Adrenaline				
Bicarbonate	20	1765.0 ± 66.0		
Tris	7	828.5 ± 66.0	53%	9.99
HEPES	10	990.0 ± 61.0	44	8.59
BICINE	6	1025.0 ± 25.0*	42	10.41
PIPES	6	1041.5 ± 62.5*	41	7.92
MOPS	8	1162.5 ± 134.5*	34	4.02
Angiotensin				
Bicarbonate	10	1710.0 ± 118.0		
BICINE	4	525.0 ± 43.5	69%	9.89
HEPES	4	562.5 ± 82.5	67	7.97
PIPES	4	587.5 ± 110.5	66	6.94
MOPS	4	600.0 ± 20.0	65	9.27
Tris	4	600.0 ± 20.5	65	9.27
Potassium				
Bicarbonate	18	1550.0 ± 74.0		
Tris	6	850.0 ± 48.0	45%	7.93
PIPES	4	900.0 ± 139.5	42	4.12
MOPS	4	1162.5 ± 128.0	25	2.62
BICINE	6	1165.0 ± 82.0*	25	3.48
HEPES	10	1305.0 ± 88.0**	16	2.13

Equipotent doses of agonists were used: adrenaline (9.70×10^{-8} M), angiotensin (4.9×10^{-9} M) potassium (4×10^{-2} M). Values are means ± s.e. mean. n = number of animals. All buffers were used in 5 mM concentration except bicarbonate (25 mM). t value represents difference from bicarbonate controls. Significantly different from * TRIS and ** PIPES.

action of the buffers on the vascular smooth muscle cells. Although some of the artificial buffers used in our study have been demonstrated to inhibit the activity of certain cellular enzymes (e.g., Ca-Mg ATPase, glutamine synthetase, monoamine oxidase and possibly some mitochondrial enzymes) in some cell types (human erythrocytes, chick nervous tissue, LM cells, rat liver) (Medzon & Gedies, 1971; Morris, 1971; Farrance & Vincenzi, 1977; Fowler, Callingham & Houslay, 1977), which could compromise cellular energy metabolism, it is unlikely that such a mechanism could account for the adverse actions of the buffers seen in the present study. One might be tempted to think that the actions of the buffers may be due to influences on tissue electrolytes, thereby causing membrane potential changes. It has been shown, however, at least for Tris, that it neither changes the concentration of intracellular sodium or potassium nor the water content of rat cardiac and skeletal muscle (Talso & Cutilletta, 1965). Although the buffers could inhibit spontaneous mechanical activity and contractility by influencing cellular pH, several arguments, which we have previously presented for this possibility for Tris (Turlapaty *et al.*, 1978), also would militate against this possibility for HEPES, MOPS, BICINE and PIPES.

Recently, it has been demonstrated that Tris buffer, in concentrations of 5 and 30 mM, inhibit Ca^{2+} uptake in both rat aortic and portal venous smooth muscle (Turlapaty, Altura & Altura, 1979a). In addition, Favalli, Chiari, Rozza & Piccinini (1977) have found that Tris lowered the Ca content of rat caudal artery. In the present study, we have observed that five different artificial buffers, including Tris, exert marked depressant effects on calcium-dependent spontaneous mechanical activity (Altura & Altura, 1974; Weiss, 1977; Altura, 1978). Collectively, these findings suggest that Tris, HEPES, MOPS, PIPES and BICINE may act directly on calcium ion exchange in vascular smooth muscles. In 1966, Good *et al.* found that synthetic organic buffers, similar to those used in the present study, exhibit some binding capacity for Ca^{2+}, Mg^{2+} and Mn^{2+}. Tris buffer has also been shown to form stable complexes with various divalent metal ions, including Ca^{2+} (Hanlon, Watt & Westhead, 1966; Allen, Baker & Gillard, 1967).

In view of the evidence obtained in this study, that Tris, HEPES, MOPS, BICINE and PIPES alter contractility and the 'true' reactivity of smooth muscles, conclusions drawn from previous studies employing these artificial buffers may need to be re-evaluated. We suggest, therefore, that at the very least, one must be cautious regarding conclusions drawn for calcium-dependent responses obtained in smooth muscles (and maybe muscles, in general) in which Tris, HEPES, MOPS, BICINE or PIPES was the sole buffering agent.

This work was supported by Research Grants HLBI-18002, HLBI-18015, MH-26236 and DA-02239 from the U.S. Public Health Service.

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(Received May 18, 1979.
Revised July 22, 1979.)