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Inotropic Influence of Macrocyclic Polyethers on Tracheal Smooth Muscle

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KOLBECK, R. C., C. LA NEAVE, A. AGUIRRE, T. M. NOSEK AND K. H. PANNELL. *Inotropic influence of macrocyclic polyethers on tracheal smooth muscle.* PHARMACOL BIOCHEM BEHAV 42(4) 645-650, 1992. – Incubated guinea pig tracheal smooth muscle exhibited both positive and negative inotropic responses to a variety of crown ether analogs that ranged in size from 12-crown-4 to 30-crown-10 and included molecules whose lipophilicity was modified by the addition of benzo- and cyclohexo- substituents on the basic molecular framework. The inotropic influence of crown ethers may not only be due to their ionophoretic capabilities but may result from their ability to affect alterations in membrane physiology.

Smooth muscle Trachea Crown ether ionophores Membrane physiology

SINCE their discovery (27,28), crown ether ionophores have experienced an ever-increasing use in many areas of organic, inorganic, and analytical chemistry. The 1987 Nobel Prize in Chemistry was awarded to Pedersen, Cram and Lehn, in part, for work related to the study of these compounds. The structural simplicity of the crown ether molecule allows a wide variety of analogs to be synthesized with varying ring sizes, numbers of oxygen atoms, ionic specificities, lipophilicity, and hydrophilicity (17,21,23,26).

The capacity of crown ethers to mimic naturally occurring ionophores in their ability to transport Na^+, K^+, and Ca^{2+} across liquid-liquid barriers (7,30,32) is of great interest in membrane physiology/pharmacology. The ability of these compounds to complex and transport metal ions is dependent upon a variety of factors (5) including the cavity/ion size ratio, the number and type of base atoms in the crown ether, the effects of electronic substituents on the crown ether, the liphophilicity of the crown ether, and the molecule's flexibility. In spite of the great potential that crown ethers have to influence the transport of ions across biological membranes, detailed studies of crown ethers as ionophores have not been undertaken. It has been suggested that crowns may function directly as facilitated carriers (7), but later noted that they could also function as ionic channels because of their ability to form aggregates within membranes (2). Crowns may also influence ion transport by altering membrane structures and/or by interacting with membrane agonist binding sites.

We previously demonstrated that analogs of 15-crown-5 alter the contractility of isolated guinea pig tracheal smooth muscle and heart muscle (19). The cavity size of 15-crown-5 led us to conclude that the contractile alterations were probably the result of changes in the transmembrane movements of Na^+ and K^+ and/or subsequent changes in intracellular concentrations of Ca^{2+}. The various substituents of the 15-crown-5 analogs used in this early work, however, were often remote from the cavity binding site and probably did little to influence the metal-ion binding constants. Interpretations of analog structure-function relationships were, therefore, difficult to make. The purpose of the present study was to build on the earlier work by including a greater variety of crown ether compounds with extensive differences in ion binding characteristics. Portions of this work have been presented before the American Chemical Society (22).

METHOD

Tracheal tissue was isolated from 350- to 450-g male Hartley guinea pigs that were killed by cervical dislocation. Following midsternal thoracotomy, tracheas were rapidly excised and placed in an oxygenated Krebs-Henseleit buffer, the composition of which was as follows (mM): NaCl, 118.0; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.1; $NaHCO_3$, 25.0, $CaCl_2$, 2.5; and dextrose, 11.0. This solution was continuously aerated with a 95% O_2–5% CO_2 gas mixture and, at 37°C, the pH was maintained at 7.35.

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Tracheal Ring Segments

The excised trachea was cleaned of excess tissue and divided into eight cylindrical segments, each 4 mm in length. These segments were selected at random to minimize regional tracheal variability [although, based upon previous work, none was expected (12)] and mounted on opposing L-shaped 30-ga needles with care being taken to preserve the epithelial lining. Each tracheal preparation was suspended in an individual water-jacketed incubation chamber containing 20 ml of the physiological medium. One of the opposing needles was anchored to the bottom of the bath, while the other was attached with 5-0 silk suture material to a Statham (Oxnard, CA) UC-3 force-displacement transducer interfaced with a UL-5 (Statham, Oxnard, CA) microscale attachment. A preload stress was applied to the tracheal rings by moving the needles apart using a myograph tension adjuster (Narco Bio-Systems, Houston, TX). Signals from the transducers were amplified and recorded on a physiological recorder (Sensor-Medics, Anaheim, CA) as grams of force. The tissue was allowed to equilibrate for 60 min under a resting tension of approximately 2 g, a tension found to achieve excellent tissue stability and responsiveness as determined by the method of Stephens and coworkers (31). This resting tension, a composite of both active and passive components, prepared the trachea to respond to the crown ethers by either relaxing or contracting.

Crown Ether Analogs

Following the 60-min equilibration period, each muscle preparation was exposed, at 37°C, to a specific crown ether. A listing of these crown ethers is found in Table 1. These crowns are not an all-inclusive group of inotropic agents but are considered in this study because of their wide variation of pore sizes and crown/ion complex stabilities. Three "crab" compounds are also included in this list since they contain ketonic groups that, in theory, assist in the ionic binding process. These ketonic groups are more basic than are the etheral oxygen atoms of the crown compounds. In the naturally occurring ionophores, for example, valinomycin, ketonic oxygen atoms are the predominant type of binding site for the complexed ions.

TABLE 1

CROWN ETHER ANALOGS USED IN THIS STUDY

Code	Name	Effective Concentration Range
A	12-Crown-4	No statistical response
B	15-Crown-5	No statistical response
C	Cyclohexano-15-Crown-5	5×10^{-5}–5×10^{-3} M
D	Benzo-15-Crown-5	5×10^{-7}–5×10^{-3} M
E	18-Crown-6	7×10^{-7}–1×10^{-4} M
F	Dicyclohexano-18-Crown-6	1×10^{-7}–1×10^{-4} M
G	Dibenzo-18-Crown-6	1×10^{-6}–1×10^{-3} M
H	Dibenzo-21-Crown-7	5×10^{-6}–1×10^{-4} M
I	21-Crown-7	No statistical response
K	Dibenzo-24-Crown-8	1×10^{-6}–1×10^{-3} M
L	Dicyclohexano-24-Crown-8	3×10^{-7}–1×10^{-3} M
M	Dicyclohexano-27-Crown-9	3×10^{-6}–1×10^{-3} M
N	Dibenzo-30-Crown-10	3×10^{-6}–1×10^{-3} M
O	Dicyclohexano-30-Crown-10	3×10^{-6}–1×10^{-3} M
P	13-Crab-4	No statistical response
Q	16-Crab-5	1×10^{-6}–1×10^{-4} M
R	19-Crab-6	7×10^{-6}–1×10^{-4} M

Concentration-Response

Log concentration-response curves were determined for each compound studied. Incremental amounts of the crown ethers were added to the baths on a cumulative basis, allowing sufficient time to lapse between successive additions for equilibrations of tension (usually less than 10 min). The contractile response of each tracheal tissue was related to corresponding control tissue—tissue that had been incubated under identical conditions but in the absence of added agents. In each experiment, the crown compounds were added until a concentration of 10^{-3} M was reached, after which time the crown analogs were repeatedly rinsed from the incubating chambers with fresh buffer. The maximal increase or decrease of tracheal tension, from the 2 g equilibrium value, was recorded and expressed as changes in tension (mN/g wet tissue weight).

Some of the crown ethers had limited solubilities in aqueous solutions. For these, primary stock solutions were made using dimethyl sulfoxide (DMSO) and subsequent experiments presented equivalent amounts of DMSO to the control tissues. The slight amount of DMSO in the incubating buffer (less than 0.1%), however, had no apparent effect on contractile properties.

RESULTS

The maximal response of the tracheal ring segments to the influence of the various crown ethers and crab compounds is indicated in Fig. 1. Some of the crown ether molecules, including 12-crown-4 (A), 15-crown-5 (B), and 21-crown-7 (I), appeared to have little or no effect on contractility, while the crowns with code letters C–H, including analogs of 15-crown-5, 18-crown-6, and 21-crown-7, caused an increase in tracheal tension. The larger crowns with code letters K–O, including derivatives of the basic units of 24-crown-8, 27-crown-9, and 30-crown-10, elicited biphasic contractile responses; at concentrations less than 10^{-5} M, these crowns caused the tracheal rings to contract, while above this concentration they caused the tracheal rings to relax. The crab compounds, 13-crab-4 (P), 16-crab-5 (Q), and 19-crab-6 (R), exerted only small positive influences on tracheal contractility over the concentration range used.

Figure 2 displays representative dose-response curves for two crown ethers, one of which enhances tracheal tension [dibenzo-18-crown-6 (G)] and one of which exhibits a "biphasic" dose-response [dibenzo-30-crown-10 (N)]. The effective concentration ranges, computed from such dose-response curves, are listed in Table 1 for each compound tested.

All contractile influences of the crown compounds were reversible, a fact indicating a lack of irreversible cytotoxicity. Figure 3 is a representative time-response curve for dicyclohexano-24-crown-8 (L) that demonstrates this reversibility. As noted, the developed tension of the tracheal ring returns to predose levels when the crown ether is rinsed from the bath.

DISCUSSION

Crown ethers are macrocyclic rings of covalently joined—(OCH_2CH_2)—units. The chemistry of these compounds is of particular interest because of their capacity to interact with

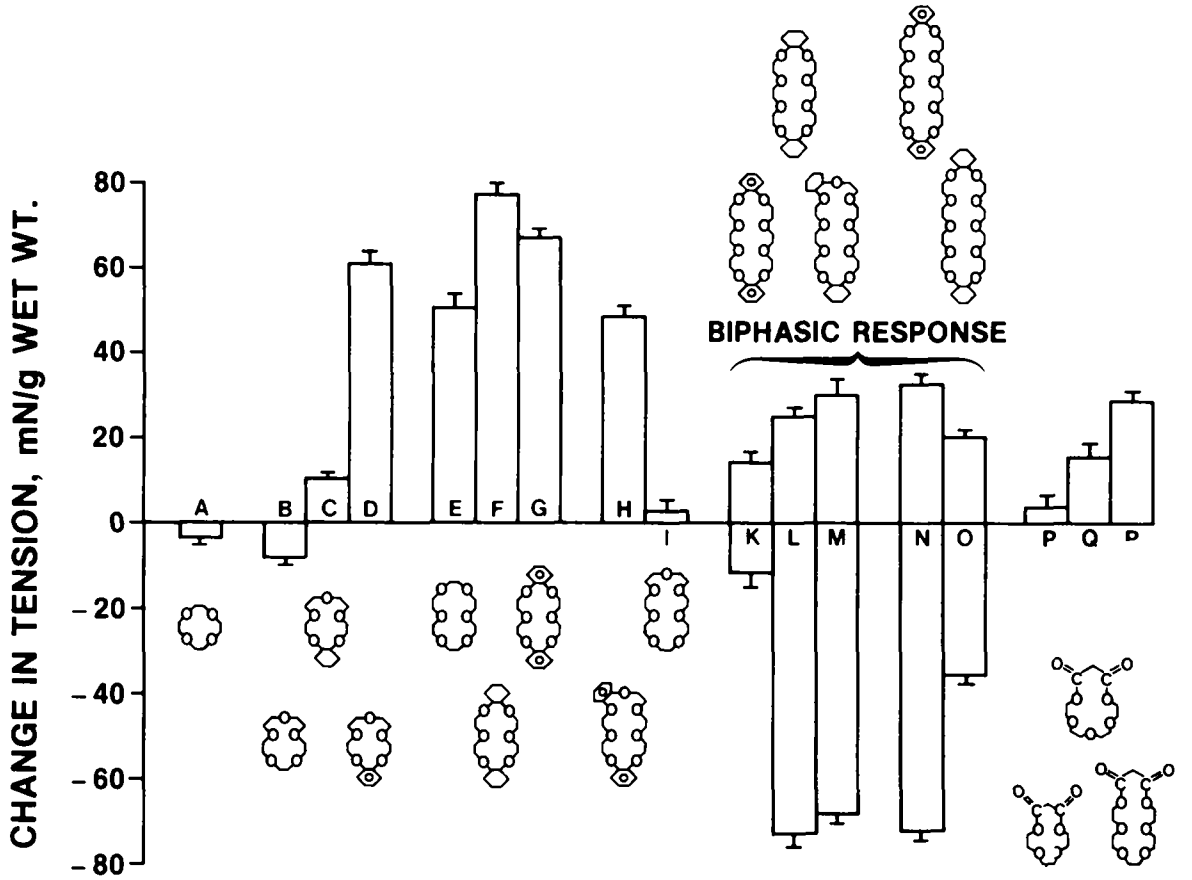

FIG. 1. Maximal increase or decrease in tension (mN/g wet wt) of incubated guinea pig tracheal rings exposed to various crown ether analogs. Control line represents a 2-g equilibration tension. The data are expressed as the mean ± SEM of 8-10 studies. The crown ether codes are identified in Table 1.

group I and group II metal ions. Accordingly, they are widely used as reagents in phase-transfer reactions, in investigations of ion-pairing phenomena, and in other reactions related to their capacity to complex and solubilize metal ions in organic solvents. Our interest in the crown ethers, however, resides in their apparent capacity to function as ionophores. Compared to analogs of naturally occurring ionophores, such as monensin (Na^+ selective), valinomycin (K^+ selective), or lasalocid acid (Ca^{2+} selective), the crown compounds are relatively easy to synthesize. The number of repeating units normally varies from 3-10, but only those containing 5 (15-crown-5) or 6 (18-crown-6) oxygen atoms in the ring have attracted widespread attention. Several excellent reviews have been published on the chemical characteristics of crown ethers, but very little information is available concerning their physiological properties (6,10,18).

A number of features are responsible for determining the strength with which a crown ether molecule interacts with an ion (5,16). These include: a) the ratio of the size of a crown ether's cavity to the size of an ion; b) the number, arrangement, and basicity of a crown ether's ring oxygen atoms; c) the steric hindrances inherent in the crown ether's molecular shape; and d) the strength with which an ion is in association with its solvent. In general, but not universally, the better the "fit" between a crown ether cavity and an ion, the higher its binding constant will be (1,25).

Most of the crown ethers used in the present study caused the tracheal smooth muscle to either contract or relax (Fig. 1). Such alterations in muscle contractility is evidence of corresponding alterations in intracellular calcium levels (9,29). Crowns can alter cellular calcium levels either "directly," by modifying the transmembrane movement of Ca^{2+}, or "indirectly," by altering the Na^+, K^+, ATPase, and/or Na^+, Ca^{2+} transport systems and, thereby, altering the transmembrane movement of Na^+ and K^+ (4,9,24).

Of the crown ethers used in this study, only three have cavity sizes that are directly compatible with the physiologically important ions, Na^+, K^+, and Ca^{2+} (Table 2). 15-Crown-5 has a ring diameter of 1.7-2.2 Å and preferentially binds Na^+ (ionic diameter 1.9 Å). 18-Crown-6 has a ring diameter of 2.6-3.2 Å and has preferential selectivity for K^+ (ionic diameter 2.7 Å). The cavity sizes of crown ethers with rings having more than 18 atoms are difficult to describe because of their many conformational isomers. 21-Crown-7, however, has a potential ring diameter of 3.4-4.3 Å and would preferentially select K^+ and Rb^+. Under appropriate conditions, 15-crown-5, 18-crown-6, and 21-crown-7 ether analogs are all capable of binding Ca^{2+} (ionic diameter 2.0 Å). In aqueous environments, however, the binding of divalent cations such as Ca^{2+} tends to be inefficient because of the high heat of hydration. The heat of hydration for Ca^{2+}, for example, has a value of 395 kcal/mol, while for K^+ the value is only 76 kcal/mol (13).

The biphasic contractile response data associated with the

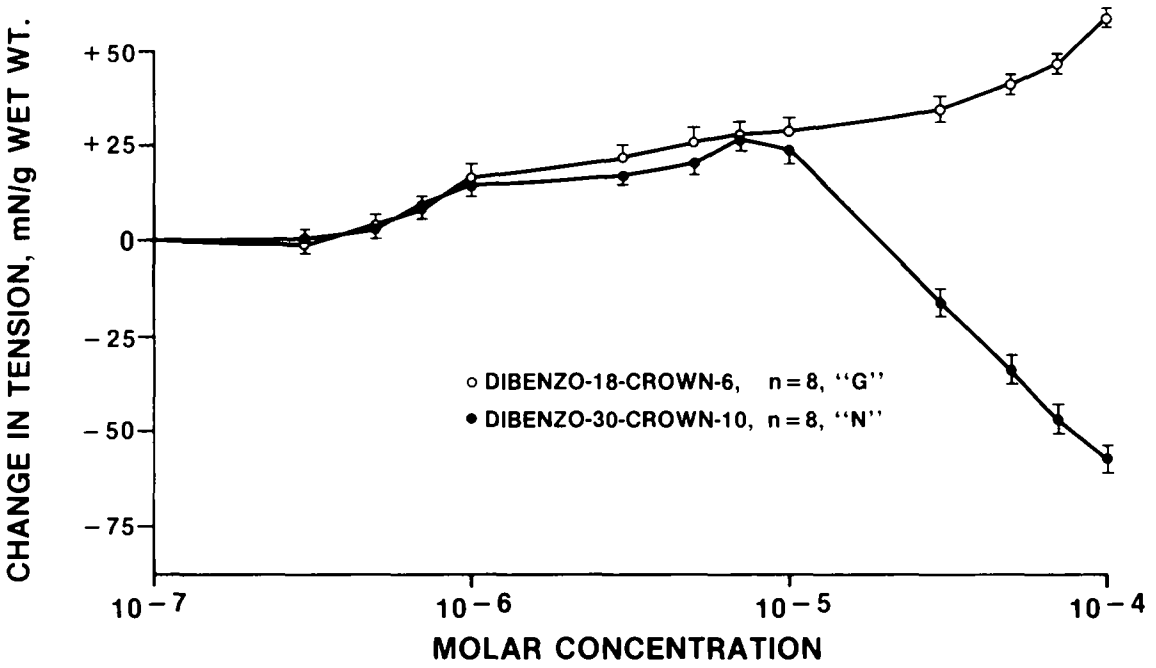

FIG. 2. Tension (mN/g wet wt) developed by incubated guinea pig tracheal rings exposed to increasing concentrations of dibenzo-18-crown-6 (G) or dibenzo-30-crown-10 (N). These dose-response curves are representative of those obtained for all compounds tested in this study, including those exerting a positive inotropic effect and those exerting a biphasic inotropic effect. The data are expressed as described in the legend of Fig. 1.

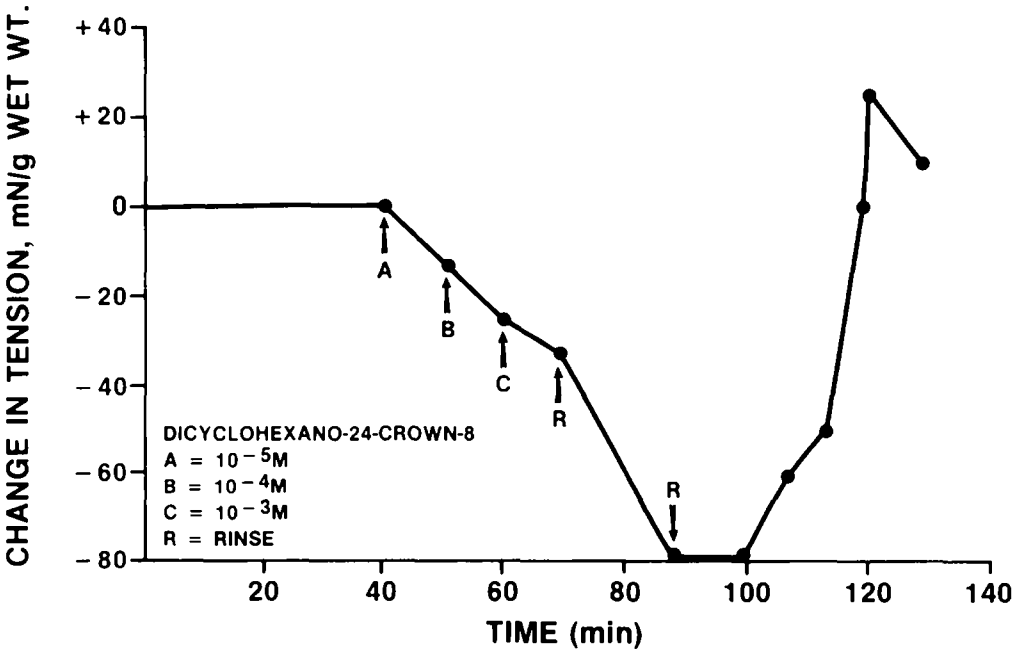

FIG. 3. Tension (mN/g wet wt) developed by incubated guinea pig tracheal rings exposed to dicyclohexano-18-crown-6. This representative dose-response curve demonstrates the reversibility, after repeated rinsing, of the influence of the crown ethers on tracheal contractile parameters. The data are expressed as described in the legend of Fig. 1.

TABLE 2

CROWN ETHER CAVITY SIZES AND LOG FORMATION CONSTANTS, log K_f

Crown ether	Cavity size	Formation Constants, log K_f		
		Na^+	K^+	Ca^{2+}
12-Crown-4	1.2–1.5 A	2.20*	1.58*	3.98†
15-Crown-5	1.7–2.2 A	3.25*	2.50–3.30*	2.36*
18-Crown-6	2.6–3.2 A	4.35*	6.10*	3.90*
18-Crown-6	2.6–3.2 A	1.80‡	2.10‡	<0.50‡
21-Crown-6	3.4–4.3 A	2.54*	4.41*	2.80*
24-Crown-8	–	2.35*	3.53*	2.66*
Dibenzo-27-Crown-9	–	1.50§	2.86§	–
Dibenzo-30-Crown-10	–	2.00*	4.60*	5.23†
Valinomycin	–	0.70*	4.90*	2.70*

Table data taken from Izatt (18).
*MeOH medium.
†Propylene carbonate, 0.1M Et_4NClO_4 medium.
‡H_2O medium.
§70% MeOH/30% H_2O medium.

larger crown ethers indicate that the relationship between a particular contractile response and some corresponding ion/ionophore geometric compatibility is undoubtedly complex. Indeed, the cavity sizes of the larger crown ethers would seem to preclude the formation of strong complexes with physiologically important cations. Busch and Truter (3), however, demonstrated that 30-crown-10 readily binds K^+ due to the flexibility of the ionophore backbone. An examination of the list of crown ether/M^+ log formation constants (log K_f) in Table 2 gives further credence to the concept that larger crowns do bind ions and may possess ionophoretic properties. K_f values, however, are very solvent dependent; the binding of K^+ to 18-crown-6 in methanol has a log K_f value of 6.10 while in water the value is 2.10. In general, the larger crown ethers exhibit a greater capacity to bind ions in nonaqueous solvents than in aqueous solvents, a characteristic apparently related to the high hydration energies necessary for the decomplexation of water prior to ion–ionophore binding. In general, the K_f values for larger crown ethers are lower than for the smaller crowns, and some K_f values measured in water are so small as to defy realistic measurement. Such information tends to suggest that larger crown ethers may be incapable of functioning as ionophores in aqueous media, but may be very effective in hydrophobic environments such as the lipid regions of biological membranes.

Based upon the above discussions, we offer three possible explanations for the inotropic behavior we observed for the crown ethers (Fig. 1).

First, the lipophilic properties of crown ethers appear to be important in determining their relative inotropic strength. Previous work by Kolbeck et al. indicates that hydrophobic substituents enhanced the inotropic effects of the crown ethers (19). Within the lipid environment of the muscle sarcolemma, crown ethers probably act as ionic traps and interrupt normal transmembrane ionic movement (14).

Second, as suggested by Behr and coworkers macrocyclic polyethers are capable of forming aggregates within lipid membranes (2). When sufficiently large, such aggregates form transmembrane bridges that function as ion-selective channels (1,2,20). The fact that the largest crown ethers used in our study exhibited the greatest inotropic effects give credence to this concept.

Third, crown ethers may modify endogenous ion channels indirectly by interacting with sites on the sarcolemma surface. In support of this idea is the remarkable reversibility of the crown ether's contractile effects (Fig. 3). It would be difficult to rationalize this rapid reversibility if the crown ethers were actually partitioning within membranes. It has been proposed that the K^+-selective ionophore dicyclohexano-18-Crown-6 alters K^+ and Na^+ permeability in the Node of Ranvier of myelinated nerves not as an ionophore but by combining to the excitable membrane in the vicinity of the K^+ permeable site and by modifying the site without affecting the activating system (1,20). The authors suggested that the crown ether probably entered the natural K^+ channel of the membrane when it was open, and therein blocked its permeability. The ability of crown ethers to alter muscle contractility, as noted in our study, may be, in part, related to such indirect alterations of K^+ movement. In a related study, it was noted that lipophilic side rings and chains also contributed significantly to the inotropic potency of the crown ethers (1,20), even though such structural features did little to alter the crown/ion formation constants. Preliminary results in our laboratory using differential scanning calorimetry have indicated that crown ethers exert a significant effect upon the surface structure of phospholipid bilayers (15).

The biphasic response noted for crown ethers coded K–O (Figs. 1 and 2) is an interesting spectacle. It has been proposed that a biphasic response may be the result of an agonist's interaction with two different types of membrane constituents, as, for example, with membrane lipids and proteins (8). It is also suggested that methylverapamil altered calcium transport by two mechanisms, a low-concentration protein-specific interaction and a high-concentration lipid effect (11). In our experiments, the larger crown ethers may also exhibit these two distinct types of interactions with the tracheal

smooth muscle membrane, both of which may be concentration dependent.

The three crab compounds studied (coded P, Q, and R) were only marginally inotropic. Clearly, the presence of the ketonic group within the cyclic framework did not impart any special properties to these potential ionophoric compounds.

To define the mechanisms by which the various crown ether analogs influence muscle contractility, future studies must make specific measurements of crown-induced alterations in cellular ionic activities.

ACKNOWLEDGEMENT

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