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COMPARISON BETWEEN BRETYLIUM AND DIPHENYLHYDANTOIN INTERACTION WITH MUCOSAL SODIUM-CHANNELS

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The antifibrillatory drug bretylium and the antiepileptic drug diphenylhydantoin cause an increase in the potential different and in the short-circuit current (SCC) across frog skin when added to the outer surface. The effect of both drugs depends upon the presence of sodium ions in the outer medium and is blocked by the specific sodium channel blocker, amiloride. Quantitative analysis shows that amiloride binds to open as well as closed mucosal sodium channel with the same affinity. The effects of diphenylhydantoin and bretylium differ with respect to their dependence on external pH. The diphenylhydantoin or the bretylium stimulatory effects are additive to the effects of oxytocin. In most cases the diphenylhydantoin and bretylium effects are also additive. It is concluded that the external side of the mucosal Na^+ channels contains sites which interact specifically with either bretylium or diphenylhydantoin and thus remove the sodium induced closure of the channels.

Introduction

Recently we have shown that bretylium an antifibrillatory drug, opens mucosal, amiloride-sensitive sodium channels [1]. In this respect bretylium was a new addition to several compounds which 'stimulate' sodium transport when applied to the external surface of the frog skin. These include diphenylhydantoin [2], amitriptyline [3], amiloride analogues [4] and more [5]. It was suggested that the action of these agents on the external surface of the epithelium is related to the well established regulatory effect of external sodium concentration on the balance between open and closed sodium channels [6]. Thus, at low external sodium concentration, when all the mucosal sodium channels are open there is no Na^+-stimulatory effect of bretylium [1] or of the amiloride analogues [4]. The effect of these external agents manifests itself only when a substantial fraction of the sodium channels are closed due to high sodium content in the mucosal side.

The aim of this study was to compare the effects of bretylium and diphenylhydantoin on the mucosal Na^+-channels. Various aspects of their action and interaction were studied. Bretylium and diphenylhydantoin were chosen because of the fact that, despite the difference in their structure and the opposite charge that they carry, they induce almost identical effects in the frog skin preparation. In addition despite the wide use of bretylium as an antifibrillatory agent [7] and diphenylhydantoin as an antiepileptic drug [8] the mechanism of their action was not elucidated yet. The study of the interaction of these drugs with the mucosal sodium channel may shed light on the mechanism of their therapeutic effects.

Materials and Methods

Experiments were performed on the abdominal skin of *Rana ridibunda*. Electrical potentials were measured through calomel electrodes connected to a high resistance input electrometer (Keithley, Model 615). The output of the electrometer was fed into a recorder (Yokogawa Electric Work Ltd. Tokyo). Current pulses of 3–5 s duration were applied to the skin through Ag-AgCl electrodes. The skin was held between two silicon rubber sheets in which holes of about 0.25 cm^2 were punched. The volume of the cell adjacent to the skin was about 0.2 ml. The skin was continuously rinsed on both sides at a rate of 0.8 ml/min. The electric resistance of the skin was measured by recording the voltage response to applications of constant current pulses. The resistance of the system without skin was also measured and was taken into account in calculating net membrane resistance. Ringer solutions contained (in mM) Na^+ 110, K^+ 2, Ca^{2+} 1, Cl^+ 114, glucose 5 and Hepes 5, at pH 7.4. Unless otherwise indicated the solution bathing the inside face contained 12 mM K^+ and 100 mM Na^+. Bretylium tosylate was obtained as powder from American Critical Care, McGaw, IL, U.S.A. Diphenylhydantoin and oxytocin were purchased from Sigma Corp. and amiloride was a gift received from Dr. L.H. Mandel, Merck Sharp and Dohme Research Laboratories, U.S.A.

Quantitative analysis of amiloride interactions with Na^+ channels. The analysis is based upon the following assumptions: (a) At high mucosal Na^+ content the equilibrium between open and closed channels, C_o and C_c, respectively, is given by the equation

$$C_o \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C_c \tag{1}$$

where k_1 and k_2 are the reaction rate constants for closing and opening the channel, respectively. Henceforth, the notation, C, C_o and C_c will denote concentrations of channels. (b) The effect of diphenylhydantoin and bretylium is to render $k_1 \ll k_2$, (see Results). (c) At a saturation effect of diphenylhydantoin or bretylium all the channels that contain the relevant sites to these drugs are open whereas without these drugs only $1/(1 + k_1/k_2)$ of channels are open. Thus if the short-circuit current (SCC) is proportional to the number of the open mucosal channels then

$$\frac{\text{SCC (with bretylium or diphenylhydantoin)}}{\text{SCC (without bretylium or diphenylhydantoin)}} = 1 + k_1/k_2$$

(d) The interaction between channels and amiloride (A) is determined by an equilibrium constant, K of the reaction

$$A + C \rightleftharpoons AC$$

where AC is the amiloride-channel complex which is always a blocked channel.

Two alternatives are considered; one in which only open channels, C_o, interact with amiloride and second in which all channels, C_o and C_c, interact with amiloride to the same extent. These two alternatives can be written as follows:

$$A + C_o \rightleftharpoons AC \tag{2a}$$

and

$$A + C \rightleftharpoons AC \tag{2b}$$

In general therefore there will be three forms of the channels C_o, C_c and CA the sum of which will be constant. C_c (see Eqn. 1) will be given by

$$C_c = C_o(k_1/k_2) \tag{3}$$

Using Eqns. 2a and 2b [AC] will be given, respectively, by

$$[AC] = C_o K [A] \tag{4a}$$

and

$$[AC] = C_o(1 + k_1/k_2) K [A] \tag{4b}$$

The total channel concentration, C_T, will be therefore either

$$C_T = C_o[(1 + k_1/k_2) + K[A]] \tag{5a}$$

or

$$C_T = C_o(1 + k_1/k_2 + (1 + k_1/k_2) K [A]) \tag{5b}$$

The ratio of concentration of open channels when

[A] = 0 to their concentration when [A] ≠ 0 is given respectively by

$$\frac{C_o(A=0)}{C_o(A)} = 1 + [A]K/(1 + k_1/k_2) \quad (6a)$$

and

$$\frac{C_o(A=0)}{C_o(A)} = 1 + [A]K \quad (6b)$$

Thus if amiloride interacts only with open channels Eqn. 6a is valid and the ratio of open channels without and with amiloride will depend on the term $(1 + k_1/k_2)$. The slope of the curve depicting $C_0(A=0)/C_o(A)$ as a function of [A] will change by the ratio of $(1 + k_1/k_2)$ values without or with bretylium or diphenylhydantoin. On the other hand if amiloride interacts with open and closed channels then (according to Eqn. 6b) the above slope should be independent of the term $(1 + k_1/k_2)$, i.e., it should be the same with or without bretylium or diphenylhydantoin.

In practice we measured SCC rather than concentration of open Na^+ channels. However, at high serosal potassium concentration (we have used 12 mM K^+ instead of 2 mM in the inside solution). SCC is limited largely by the permeability to sodium of the mucosal membranes [9] and thus the ratio of SCC (A = 0)/SCC(A) represents approximately the term $C_0(A=0)/C_o(A)$.

Results

(1) Comparison of the Natriferic responses of bretylium and diphenylhydantoin

Table I represents analysis of single responses to application of bretylium or diphenylhydantoin to the external surface of frog skins.

The analysis is shown for drug concentrations that were above the EC_{50}. From these results it can be seen that the responses to bretylium and diphenylhydantoin are similar in terms of the extent of increase in SCC and potential difference. For both compounds, the increase in current is always greater than the increase in potential difference, i.e., the response is associated with an increase in membrane conductance. The EC_{50} for bretylium was about 1 mM and for diphenylhydantoin it was more variable, with the limits being 0.05–0.15 mM.

The kinetics of the responses were evaluated by measuring the time taken to reach 50% of the response in the 'on' and the 'off' stages. For the 'on' response the time was 53 ± 5.4 (S.E., $n = 14$) and 96 ± 15 (S.E., $n = 14$) seconds for bretylium and diphenylhydantoin, respectively. This difference is not significant statistically. For the 'off' response the time was 88 ± 49 (S.E., $n = 14$) and 365 ± 88 (S.E., $n = 14$) seconds for bretylium and diphenylhydantoin, respectively. The difference in the time of the 'off' response is significant at < 0.02 level.

The similarity of the time of the 'on' response

TABLE I

STIMULATORY EFFECTS OF BRETYLIUM AND DIPHENYLHYDANTOIN WHEN APPLIED TO THE EXTERNAL SURFACE OF FROG SKINS

The average response and standard error of increase in potential difference (ΔV), in short circuit current (ΔSCC) and in the percent increase in those parameters are indicated. The number in parenthesis indicates the number of experiments.

Drug added	Concn. (mM)	ΔV (mV)		% increase in V	ΔSCC ($\mu A/cm^2$)	% increase in SCC
Bretylium	1.2	15.6 ± 2.3	(4)	26 ± 1.9	25.0 ± 6.0	63 ± 5.7
Bretylium	2.4	18.2 ± 2.0	(7)	34 ± 4	44.0 ± 11.5	83 ± 14.7
Bretylium	3.6	28 ± 3.5	(3)	48 ± 10	72.0 ± 13.0	163 ± 15.3
Diphenylhydantoin	0.18	14.9 ± 2.6	(8)	23 ± 2.7	39.0 ± 7.0	63 ± 10.7
Diphenylhydantoin	0.36	16.5 ± 3.1	(10)	29 ± 3.1	43.0 ± 12.0	60 ± 10.1
Diphenylhydantoin	0.72	21.0 ± 2.2	(9)	33 ± 3.0	51.5 ± 13.5	80 ± 13.0

may indicate that this time is determined by diffusion of the drugs through unstirred layers to the responsive sites. On the other hand, longer 'off' response for diphenylhydantoin signifies a real slower off rate constant for this drug. Since the EC_{50} of diphenylhydantoin is about 10-times lower than that of bretylium the above results indicate that at least part of the lower EC_{50} of diphenylhydantoin is related to slower off rate constant of this drug.

(2) Sodium dependence and amiloride sensitivity of the bretylium and diphenylhydantoin effects

Table II shows that the dependence of the diphenylhydantoin effect on external Na^+ concentration is similar to those of bretylium. Thus, the neutral diphenylhydantoin (or the anionic diphenylhydantoin moiety) acts like the familiar organic cations in releasing the Na^+ self inhibition of the channels [5]. It has already been shown that the stimulation effects of these drugs are sensitive to amiloride [1,2]. A careful analysis shows that the plot of the ratio between the SCC in the absence of amiloride and the SCC in the presence of amiloride as a function of amiloride concentrations yields straight lines which are similar with or without the external agents diphenylhydantoin or bretylium (Fig. 1). As explained above these findings are consistent with the assumption that amiloride interacts similarly with the open and closed (i.e., Na^+ self-inhibited) Na^+ channels. Thus the effects of the cation bretylium and the neutral or anionic diphenylhydantoin are similar also in terms of their sensitivity to amiloride.

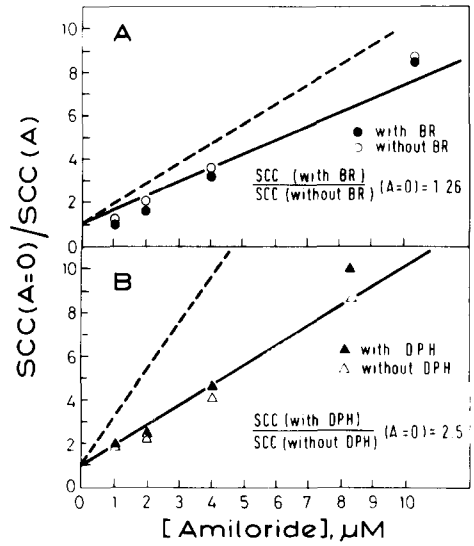

Fig. 1. Analysis of the interaction of amiloride with the Na^+ channel. The relationship between the ratio of SCC without amiloride, SCC (A = 0), to that in the presence of amiloride, SCC (A), as a function of amiloride concentrations in the presence (full symbols) and absence (empty symbols) of bretylium (A) or diphenylhydantoin (B) is shown. The dashed lines represent the theoretical curve expected for bretylium or diphenylhydantoin if amiloride were bound only to the open configuration of the sodium channel. The slope of the dashed line is increased by the ratio of SCC (with drug)/SCC (without drug) at zero amiloride relative to the slope of the full line. Only the amiloride sensitive SCC was taken into account for these calculations. This was determined by subtracting from the measured SCC the amount that was measured in the presence of 50 μM amiloride. The actual figures of amiloride sensitive SCC were 75 and 45 $\mu A/cm^2$ for Panels A and B, respectively. The amiloride insensitive SCC was in both cases about 20 $\mu A/cm^2$ and was not stimulated by either bretylium or diphenylhydantoin.

TABLE II

EFFECT OF MUCOSAL $[Na^+]$ ON RESPONSE TO BRETYLIUM AND DIPHENYLHYDANTOIN

Average values and standard errors are shown. The number of experiments is indicated in parenthesis. Low $[Na^+]$ was achieved by replacing Na^+ with K^+ or choline. The concentrations of the drugs were 2.4 and 0.72 mM for bretylium and diphenylhydantoin, respectively.

$[Na^+]_{out}$ (mM)	Bretylium		Diphenylhydantoin	
	% increase in SCC	% increase in V	% increase in SCC	% increase in V
110	98 ± 22	43 ± 7 (4)	120 ± 41	53 ± 14 (8)
55	59 ± 25	26 ± 9 (3)	75 ± 24	31 ± 8 (5)
33	63	18 (1)	51	21 (1)
11	21 ± 13	9 ± 6 (4)	35 ± 18	13 ± 6 (7)
0	0	0 (2)	0	0 (6)

Another point which emerges from these experiments is that both bretylium and diphenylhydantoin are unable to activate the amiloride insensitive part of the SCC. The portion of the amiloride resistant SCC can be as high as 35% of the total SCC (see legend of Fig. 1).

(3) Bretylium and diphenylhydantoin effects are additive to the effects of oxytocin

Oxytocin is a hormone which regulates Na^+ transport in NaCl transporting tight epithelia [9,10]. It is well established that this hormone increases the number of sodium channels in epithelia and that this effect is mediated by the activation of adenylate cyclase in these cells [11]. As can be seen in the representative experiment shown in Fig. 2, the responses to bretylium and diphenylhydantoin are additive to that of oxytocin, which was applied to the internal surface of the skin. This result is in agreement with earlier investigation on diphenylhydantoin [2].

Quantitatively, the diphenylhydantoin or bretylium induced increments in SCC, before and after oxytocin, are percentagewise on the average about the same. Since the effect of oxytocin is to increase the number of mucosal sodium channels [9] the simple interpretation of the above results is that the newly appearing oxytocin-induced Na^+-channels are sensitive to Na^+, bretylium and diphenylhydantoin to the same degree, as the previously present channels.

(4) pH dependence of the diphenylhydantoin and bretylium effects

The experiments described so far do not provide a clear feature that can discriminate between bretylium and diphenylhydantoin effects on the mucosal membranes of NaCl transporting epithelia, except by the range of concentration required to obtain a particular response.

An obvious experiment called for in this case is the study of pH dependence of the bretylium and diphenylhydantoin stimulatory effects. The pH should affect the amount of charges on the mucosal membrane surface as well as the extent of ionization of diphenylhydantoin ($pK_a = 8.3$–9.2) [12,13]) and thus should differentiate between these two compounds. Qualitatively, this general expectation was indeed seen at least for concentrations which are around the EC_{50} for these compounds (Fig. 3). Thus the bretylium effect increases with increased pH whereas the diphenylhydantoin effect decreases.

More experiments are needed to establish whether the diphenylhydantoin neutral or the diphenylhydantoin anionic molecule is the active element. In the context of this article it is concluded that the study of the pH sensitivity of the stimulatory effects of bretylium and diphenylhydantoin does not indicate a difference in the enti-

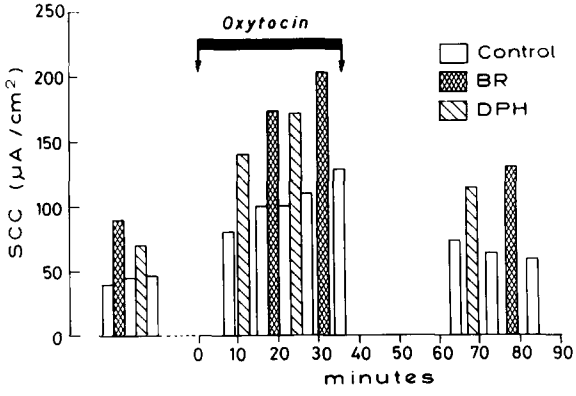

Fig. 2. Additivity of oxytocin and bretylium or diphenylhydantoin effects. The stimulation of SCC by bretylium (2.4 mM) or diphenylhydantoin (0.36 mM) in the outside solution before, during and after application of oxytocin (60 mU/ml) to the internal solution is shown. The blank columns represent control values of SCC without any drug present in the outside solution.

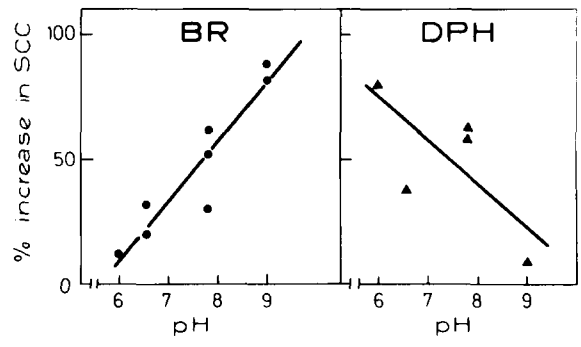

Fig. 3. Effect of external pH on percent increase of SCC caused by bretylium (BR) or diphenylhydantoin (DPH). Bretylium (1.2 mM) or diphenylhydantoin (0.18 mM) were added to the solution facing external surface of a frog skin. Each point represents a separate experiment conducted on a different frog skin preparation.

ties acted upon by these drugs. The differential effect of pH as expressed in Fig. 3 could be accounted for by a change in the electric interfacial fields produced through the pH effects on ionization of external membrane proteins. This factor will affect differentially the concentration of the drugs near the membrane relative to their concentration in the bathing fluid.

(5) Are the effects of bretylium and diphenylhydantoin additive?

A central question of this presentation is whether bretylium and diphenylhydantoin act on different Na^+-channels or on the same channels. Since the two drugs are structurally different and carry a different charge it is quite unlikely that they act on the same site (except for a remote speculation mentioned in the Discussion). The above question reduces to the problem of whether a channel can have either a diphenylhydantoin or a bretylium site only or whether each channel can contain both sites. The answer to this question depends on the type of interactions between these two compounds when applied together to the outer surface of the frog skin.

Table III represents results of four experiments

TABLE III

ADDITIVITY OF THE STIMULATORY EFFECTS OF BRETYLIUM AND DIPHENYLHYDANTOIN

Four samples of experiments (A, B, C, and D) are shown. The drugs were applied intermittently to the external surface of the frog skin for a period of 3–5 min until a steady-state short-circuit current (SCC) was attained. After each drug application the external surface was rinsed with drug-free solution until a steady state of short-circuit current was attained. The value of short-circuit current between drug applications are listed only if they varied by more than 5% from the previous value.

(A) Drug added (mM)		SCC ($\mu A/cm^2$)	(B) Drug added (mM)		SCC ($\mu A/cm^2$)
Diphenyl-hydantoin	Bretylium		Diphenyl-hydantoin	Bretylium	
–	–	64	–	–	60
–	1.2	84	0.36	–	88
–	2.4	84	–	–	75
–	3.6	104	0.18	–	84
0.72	3.6	160	0.72	–	108
–	–	96	–	–	104
0.18	–	128	0.72	3.6	152
0.36	–	122	–	–	112
0.72	–	126	–	1.2	136
0.72	3.6	154	–	2.4	161
			–	3.6	164
			0.72	3.6	160
(C) Drug added (mM)		SCC ($\mu A/cm^2$)	(D) Drug added (mM)		SCC ($\mu A/cm^2$)
Diphenyl-hydantoin	Bretylium		Diphenyl-hydantoin	Bretylium	
–	–	44	–	–	52
0.36	–	100	–	2.4	118
0.72	–	98	–	3.6	169
0.72	3.6	126	0.18	3.6	189
–	–	32	–	–	56
–	2.4	68	0.18	–	89
–	3.6	78	–	7.2	144
0.72	3.6	100	0.18	7.2	144
			0.54	7.2	164
			0.54	–	114
			–	–	59

in which interactions between diphenylhydantoin and bretylium were studied. These are typical samples of 15 experiments that illustrate the type of variability observed. Part A depicts results of an experiment which indicates that diphenylhydantoin and bretylium effects are almost completely additive. Part B illustrates experiments which show that adding bretylium to diphenylhydantoin causes a clear increment in SCC but much less than expected for a completely additive response. Moreover, in the same experiments addition of diphenylhydantoin to bretylium did not lead to any further increase in the response. The two other parts (C and D) demonstrate a clear partial additivity of diphenylhydantoin and bretylium effects. The data can be summarized by stating that there are epithelia in which the bretylium-diphenylhydantoin effects were completely additive; in others bretylium-diphenylhydantoin responses seem to be only partially additive and in a few cases no sign of additivity was observed when diphenylhydantoin was added to bretylium. There was no exception to the rule that addition of bretylium to diphenylhydantoin causes an increments in SCC.

An expression of additivity in SCC depends upon the condition that the basolateral aspect of epithelial cells does not constitute a limiting factor for the SCC. To approach this condition, we have used high K^+ concentration in the internal solution. However, it is impossible to rule out some basolateral limitation of the SCC. Thus, even partial additivity as judged by increments in SCC can result from complete additivity in terms of mucosal sodium permeability.

It is concluded, therefore, that there are channels which have on their external aspect either a bretylium site or a diphenylhydantoin site only. From the data presented here the possibility that there are channels which contain on their outer surface both a bretylium and a diphenylhydantoin site together can not be ruled out.

Discussion

The sodium permeability of mucosal membranes of tight epithelia is known to be affected by hormones [9,10,14], ions [6] and drugs [1–5]. There are two distinct mechanisms for regulating the sodium permeability through determination of the number of open channels in the membrane: (1) a mechanism which involves the protoplasmic side of the mucosal membrane; (2) a mechanism which affects the external side of the mucosal membrane. Typical examples for the first mechanism are the hormones oxytocin and aldosterone which through increase of cellular cAMP [11] or induction of specific protein synthesis [14], respectively, increase the number of sodium channels in the mucosal membrane.

The corner-stone for the second mechanism is the Na^+ self-inhibition of Na^+ permeability of mucosal membrane [6] which was shown to be caused by a change in the concentration of open channels [16] (and not by a change in conductivity of single channels). This Na^+ self-inhibition can be removed by several 'external' drugs including bretylium and diphenylhydantoin [1,5].

It is clear that the two mechanisms are independent. Thus, oxytocin and diphenylhydantoin or bretylium induced increase in SCC are entirely additive. Moreover, the fact that percentagewise the stimulatory effect of bretylium or diphenylhydantoin remains the same after oxytocin, suggests that the oxytocin induced sodium channels are similar in their sensitivity to Na^+ self-inhibition and to removal of the inhibition by the external drugs.

Many of the external compounds which remove Na^+ self-inhibition constitute a group of organic cations. The starting point for this study was the exceptional position of diphenylhydantoin as an external agent since this compound at physiological pH values is either a neutral or an anionic compound. The question that arose was whether this compound can still act on the same site as the other agents. One possibility which was considered was that a form of cation (diphenylhydantoin M^{2+}), where M^{2+} stands for Ca^{2+} or Mg^{2+}, was the active agent. However, the clear additivity of the stimulatory effects of bretylium and diphenylhydantoin as demonstrated in this study remove the necessity to look for an unknown diphenylhydantoin complex which will conform to the group of organic cations. This study indicates that diphenylhydantoin and bretylium sites are different, and that some if not all of the sodium channels contain only one of these sites.

The fact that not all the channels contain a bretylium or a diphenylhydantoin site is consistent also with observations on the extent of Na^+ self-inhibition. From the work of Van Driessche and Lindemann [16], one may assume that at high external [Na^+] at least 85% of the channels are closed. If this inhibition could be removed by saturating concentrations of bretylium or diphenylhydantoin, one would expect to find up to 550% increase in Na^+ permeability. The most that we encountered in terms of percent increase in SCC at high bretylium (3.6 mM) was 230% and at high diphenylhydantoin (0.72 mM) was 150% (see also Table I). Thus, even the sum of highest responses to bretylium and diphenylhydantoin observed in our experiments falls short of the expected increase in mucosal sodium permeability if the entire Na^+ self-inhibition were removed. These considerations lead to the conclusion of the existence of Na^+ channels whose self-inhibition can not be removed by either bretylium or diphenylhydantoin. Consequently it is possible to suspect that the other external organic cation agents (i.e., amitriptyline and amiloride analogues, etc.) may not constitute a homogeneous group which act on a single site on the sodium channel. It is possible that the external aspect of the mucosal sodium channels contains several types of sites which when combined with specific compounds can remove the Na^+ self-inhibition. According to this possibility, the fact that bretylium and diphenylhydantoin effects were additive is not exceptional and there may be other compounds which will activate Na^+ channels that are insensitive to both bretylium and diphenylhydantoin.

As mentioned in the introduction the possibility that the effects of bretylium and diphenylhydantoin on mucosal type Na^+ channels may be involved in their therapeutic effects should be considered. Bretylium which has antifibrillatory activity in animals and humans [7] was shown to stimulate and inhibit catecholamine release [17]. However, these effects are apparently not related to its antifibrillatory activity [18].

Diphenylhydantoin which is the most commonly used antiepileptic drug [8] has been shown to block or decrease Na^+ current through the 'action potential' Na^+ channels [19,20], inhibit neurotransmitter release [21,22] and stimulate ($Na^+ + K^+$)-ATPase activity [23]. It is not clear howeer which of these mechanisms if any is responsible for its antiepileptic effect.

The results presented in this study as well as previous publications [1,2] suggest an investigation of the sensitivity of the therapeutic effects of these drugs to amiloride.

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