Biochimica et Biophysica Acta, 693 (1982) 503–506
Elsevier Biomedical Press

BBA Report

BBA 70075

BRETYLIUM OPENS MUCOSAL AMILORIDE-SENSITIVE SODIUM CHANNELS

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(Received June 21st, 1982)

Key words: Ion channel; Bretylium; Amiloride; Na^+ channel; (Frog skin)

Addition of the quanternary ammonium compound, bretylium, to the outer surface of a frog skin leads to an increase in the potential difference and in the short circuit current across the skin. Bretylium does not have any effect when applied to the inside face of the frog skin. The effect of bretylium is dependent upon the presence of sodium ions in the outer medium; it is depressed when sodium is replaced by choline or potassium but not when lithium substitutes for sodium. The bretylium effect is blocked by the specific sodium channel blocker, amiloride. It is proposed that bretylium opens mucosal, amiloride-sensitive sodium channels.

Bretylium, a quanternary ammonium compound is known for its antifibrillatory activity in animals and humans [1,2]. Although extensively studied [1–4] the molecular mechanism responsible for its pharmacological effect has not been established as yet. The compound is also known to stimulate and inhibit catecholamine release [5,6]. However, these effects are apparently not related to its antifibrillatory activity [6,7]. In the course of a study aimed at elucidating the mechanism of its action, we found that it has a specific action on the outer membrane of amphibian skins. We report here the main features of its action on this epithelium. Similar effects were observed on the toad bladder epithelium.

Experiments were performed on the abdominal skin of *Rana pipiens.* Skins were held between two silicon rubber rings and the electrical membrane potential across the skin (pd) and membrane resistance were monitored using conventional techniques. (For more details see figure legends).

Fig. 1 shows that bretylium tosylate when applied to the outer surface of a frog skin causes a sustained reversible increase in the potential difference across the skin. This increase in membrane potential is accompanied by a small increase in conductance. The increase in potential difference is highly reproducible; of more than 50 trials of bretylium applications in the concentration range of 0.5 to 5 mM we never encountered a preparation which failed to respond in a typical way. In 25 trials of application of 2.5 mM bretylium the percent increase in the potential difference across the skin was $45.6 \pm 2\%$ (S.E.). The concomitant change in membrane conductance and in short circuit current was $18 \pm 2.3\%$ and $78 \pm 5.8\%$ ($n = 9$), respectively. A dose-response curve for a particular preparation is shown in Fig. 2A. An EC_{50} of approx. 1 mM can be estimated for the bretylium effect on the mucosal side of the epithelial cells. Bretylium has no effect on the potential difference or on the conductance when applied to the inside face of the skin (Fig. 1).

The following lines of evidence indicate that bretylium acts by increasing the permeability of the mucosal membranes to Na^+: (1) The effect of bretylium is depressed when sodium ions are replaced by choline (Fig. 2B) or potassium ions (data not shown). On the other hand bretylium induces increase in potential difference and in

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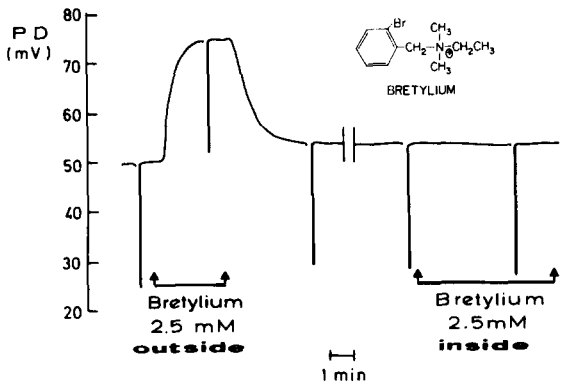

Fig. 1. Effect of bretylium on the potential difference across frog skin. Electrical potentials were measured through calomel electrodes connected to a high resistance input electrometer (Keithley, Model 615). The output of the electrometer was fed into a recorder (Yokogawa Electric Work Ltd. Tokyo). Current pulses of 3–5 s duration were applied to the skin through Ag-AgCl electrodes. Potential changes in response to application of 2 μA pulses are shown. The skin was held between two silicon rubber sheets in which holes of about 0.25 cm^2 were punched. The volume of the pipe cell close 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 resistance of the system without the frog was such that passage of 2 μA gave a pulse response of 11 mV. This figure was subtracted from the recorded response in order to calculate the resistance of the frog skin. Ringer solutions contained (in mM) Na^+ 110, K^+ 2, Ca^{2+} 1, Cl^- 114, glucose 5 and Hepes 5, pH was 7.2. Bretylium tosylate was obtained as powder from American Critical Care, McGaw, IL. Tosylate (4-toluene sulfonate) at 3 mM did not have any effect on the skin.

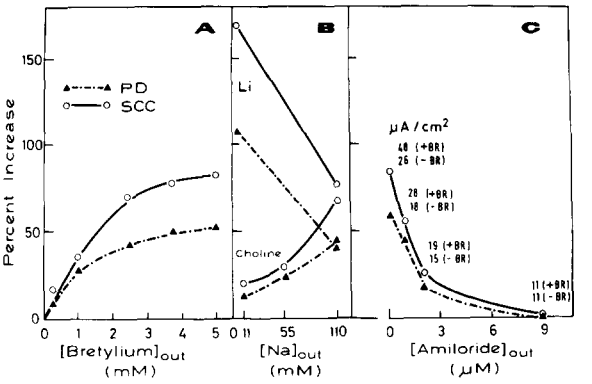

Fig. 2. Dose response curve for a bretylium-induced increase in membrane potential difference across frog skin and its sensitivity to Na^+ and amiloride concentrations. (A) Percent increase in potential difference (pd) and in short circuit current (scc) caused by adding different concentrations of bretylium to the outer medium in a single frog skin preparation. After each response to a particular bretylium concentration (as shown in Fig. 1) the outer surface was rinsed with normal Ringer before applying the next bretylium concentration. Short circuit current determined by dividing potential difference pd with membrane resistance. (B) Percent increase in potential difference and scc by the addition of 2.5 mM bretylium to the outer medium as function of Na^+ content. Na^+ was replaced by choline$^+$ or by Li^+. The solution on the inner side of the frog skin was always normal frog Ringer. The Ringer solution on the outer face was first replaced by a low Na^+ solution and then 2.5 mM bretylium was added in the same low sodium medium. The experiments with choline$^+$ and Li^+ were done on different frog skin preparations. (C) Percent increase in potential difference pd and scc caused by the addition of 2.5 mM bretylium to the outer medium at various amiloride concentrations. The solution of the outer face was first replaced by a Ringer solution containing a given concentration of amiloride. After achieving a steady potential, 2.5 mM bretylium was added in a Ringer containing the same concentration of amiloride. Numbers above points denote scc in $\mu A/cm^2$ with and without bretylium in the presence of (from left to right) 0, 0.9, 2.0 and 9.0 μM amiloride.

membrane conductance when lithium is substituted for sodium. The choline-Na^+ experiment (Fig. 2B) is representative of five experiments done on different frog skin preparations which demonstrated invariably that replacement of Na^+ by choline markedly reduced the effect of bretylium. The Li^+-Na^+ curves (Fig. 2B) are representatives of three experiments all of which indicated that the responses to bretylium in Na^+-substituted lithium Ringer is the same or higher than in a normal frog Ringer. (2) The bretylium effect is antagonized by the specific channel blocker, amiloride (Fig. 2C). Thus, in the presence of 9 μM amiloride in the outer medium no increment, in potential difference or in membrane conductance could be observed by the addition of bretylium. If it is assumed that the residual short circuit current measured in the presence of 9 μM amiloride represents an amiloride insensitive component, then it can be gathered from the numbers appearing in Fig. 2C that 0.9 μM amiloride produces a reduction of about 52 and 57 percent of the current in the absence or presence, respectively, of bretylium. It can be concluded therefore that the sensitivity of the bretylium-induced increase in Na^+ permeability to amiloride is about the same as that of the innate amiloride-sensitive sodium transport system. A detailed study of the dependence of the sensitivity to amiloride upon sodium and bretylium concentrations is now in progress.

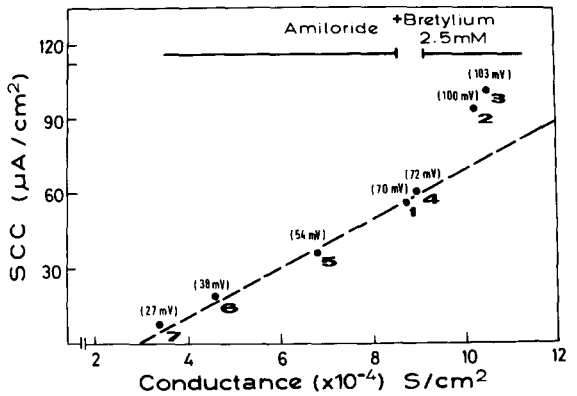

Fig. 3. Relationship between membrane short circuit current and membrane conductance. The figure in parenthesis denotes the potential difference at zero current. Point 1 denotes first reading taken when both sides of the membrane contained normal frog Ringer. Points 2 and 3 were taken close to the plateau level of the response to the outside application of 2.5 mM bretylium. Point 4 taken close to complete recovery from the bretylium effect. Points 5, 6 and 7 determined in course of the response of the membrane to outside application of 10^{-4} M amiloride.

Fig. 3 shows the relationship between membrane conductance and short circuit current in a single frog skin preparation. The points were taken during the time course of the epithelial response to successive applications of bretylium and amiloride to the outer membrane. For a simple model of the frog skin [8,9], if the only variable is the mucosal permeability of Na^+, a linear relationship between overall membrane conductance and short circuit current is expected. The slope of such a line expresses the sum of the electromotive forces operating at the mucosal ('Na battery') and basolateral ('K battery' and 'electrogenic Na^+-pump') membranes [10]. It is clear that for the example shown in Fig. 3 the current increment due to bretylium is higher than expected from the concomitant change in conductance. This can be accounted for by an increase in the electromotive force of the electrogenic Na^+ pump, secondary to an increase in intracellular Na^+ concentration [11,12].

The possibility that the bretylium effect on the Na^+ channels is related to an Na^+ driven accumulation of bretylium in presynaptic adrenergic neurons [13,14] should be considered. Put in another form: do we observe in the mucosal surface of the frog skin an Na^+-dependent bretylium uptake which due to the positive charge of bretylium expresses itself as an increase in Na^+ permeability? Two factors, however, are not in accord with this possibility: (1) Na^+-mediated cotransport at nerve terminals is insensitive to amiloride (Kanner, B.I., personal communication) whereas the bretylium effect on frog skin is completely blocked by amiloride (Fig. 2C); (2) Replacement of Na^+ by Li^+ abolishes the uptake of transmitters at nerve endings [15] but does not interfere with the response to bretylium (Fig. 2B).

The bretylium-induced increase in Na^+ permeability can be caused in principle either by increase in number of open Na^+ channels or by increase in conductivity of single Na^+ channels [16]. A direct answer to this question awaits a noise analysis under bretylium and amiloride. It is interesting to note however, that the dependence of the number of open mucosal Na^+ channels upon Na^+-concentration as determined by noise analysis [17] and by determining saturable binding sites to a potent amiloride analogue [18] corresponds roughly to Na^+ sensitivity of the bretylium effect (Fig. 2B). Thus the decrease of bretylium effect at low Na^+ concentrations may result from the increase in the number of open channels (i.e. the decrease in the number of silent, closed channels). Such a mechanism was already suggested for the stimulatory rather than inhibitory effects on sodium transport of some amiloride analogues [19,20]. Furthermore, the molecular characterization of these sodium stimulants as having 'a hydrophobic end and a positive-charge end' [19] apply equally well to bretylium.

The bretylium induced increase in membrane potential is not unique for frog skin. We observed the same effect using toad skin and toad bladder preparations. Thus the effect seems to be general for mucosal membranes. Furthermore, preliminary studies carried on 3T3 mouse fibroblasts show that bretylium induces depolarization of the plasma membrane potential, as determined by the lipophilic cation distribution method [21]. This depolarization is partially blocked by amiloride and is dependent on the presence of external Na^+. Thus, bretylium induced increase in Na^+ permeability may be a general phenomenon in cells which contain silent mucosal type Na^+ channels in their plasma membrane.

Is the increase in Na^+ permeability induced by bretylium the underlying factor responsible for its antifibrillatory effect? An extensive study of bretylium effects on Na^+ leak and content in heart cells and of the sensitivity to amiloride of its effects on the heart should answer the above question. These experiments are now in progress.

Bretylium and amiloride seem to be analogues of veratridine and tetrodotoxin as openers and blockers of mucosal and voltage-dependent axonal sodium channels, respectively. As such, bretylium may serve as an important tool for detecting the presence of silent sodium channels in cell membranes.

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