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A FOOD DYE, ERYTHROSINE B, INCREASES MEMBRANE PERMEABILITY TO CALCIUM AND OTHER IONS

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A widely used food additive erythrosine B, which has been implicated in minimal brain dysfunction in children was examined for its ability to increase membrane permeability to calcium ions. Planar phospholipid bilayer membranes become permeable to calcium, potassium and chloride ions when erythrosine B is added to the aqueous phase at concentrations which were used by others to demonstrate effects on neuromuscular preparations. The observed increase in permeability to Ca^{2+} was of sufficient magnitude that equivalent effects on cells would seriously tax the systems which maintain low cytoplasmic Ca^{2+} levels. The permeability increase in the lipid bilayer membrane is time dependent and increases with erythrosine B concentration raised to a high power (4 to 7). This indicates that the permeability pathway is generated by the cooperative action of a number of erythrosine molecules. This permeability increases dramatically with increasing transmembrane voltage indicating that cells or organelles bearing potentials across their membranes should be particularly sensitive to the dye. We propose that the neurological effects of erythrosine stem from the increased Ca^{2+} permeability.

Introduction

FD and C red No. 3, erythrosine B (2′, 4′, 5′, 7′-tetraiodofluorescein), is a food dye widely used in such items as candy, sherbert, meat, bakery goods and maraschino cherries. It is one of the coloring agents officially sanctioned for use in foods by the Food and Drug Act of 1906. Despite years of testing in the laboratory and in the human population, recent findings [1,2] have raised serious doubts about the safety of food dyes including erythrosine. One of the most striking results was reported by Augustine and Levitan [3] and Augustine [4]. The application of erythrosine to frog neuromuscular synapses increased acetylcholine release in a dose-dependent fashion.

The effects of erythrosine at the frog neuromuscular junction were reminiscent of the action of a protein isolated from black widow spider venum [5,6]. The venum was shown [7] to have the ability of forming ion-conductive channels in lipid bilayer membranes which allowed Ca^{2+} to cross the membrane. According to current theories this is sufficient to cause synaptic vesicle fusion and transmitter release. Augustine and Levitan [3] have reported that the removal of Ca^{2+} from the medium and addition of EGTA did not prevent the erythrosine effect. However, release of Ca^{2+} from internal stores by the dye could still occur. This source of Ca^{2+} has been proposed by others [8,9]to explain the induction of transmitter or hormone release by elevating cytoplasmic sodium ion concentration.

We have explored the possibility that erythrosine simply acts as an ionophore allowing Ca^{2+} to cross membranes. This paper describes experiments which show that erythrosine has the ability to conduct ions including Ca^{2+} across lipid bilayer membranes.

Materials and Methods

Measurement of permeability changes

Permeability changes were measured electronically in terms of conductance changes, i.e. the flow of ions through the membrane in response to an externally applied voltage. The membrane whose permeability was changed was a phospholipid bilayer. The planar lipid bilayers were made according to the method developed by Montal and Mueller [10] and as described previously [11]. The hole in the saran wrap partition which served to support the membrane, was 0.15 mm in diameter. Soybean phospholipids purified according to the method of Kagawa and Racker [12] were used to make the membrane. All experiments were performed under voltage clamp conditions using an operational amplifier in the inverted mode (as previously described [11]). Calomel electrodes were used to interface the electrical circuitry with the solution.

In the absence of added erythrosine the permeability of the membrane to ions was very low ($<10^{-11}$ S in conductance). Measurements were only made when the dye induced permeability was greater than ten times the native membrane permeability.

Diphytanoylphosphatidylcholine was purchased from Avanti Biochemicals Inc., Birmingham, AL.

Measurements with ion-exchange membranes

In order to determine the ratios of ion permeability via the Goldman-Hodgkin-Katz equation (Hodgkin and Katz [18]) it is necessary to convert ion concentrations into activities. Since the activity of each ion in an ion pair can be different one would like to have a single ion activity measurement. Unfortunately, only salt activities are available from the literature. Single ion activity ratios can be obtained using ion exchange membranes. (Ionac Chemical Co., division of Sybron Corp., Birmingham, NJ; MC 3 142 cation exchanger, MA 3 148 anion exchanger). By using a cation exchange membrane to separate solutions of different salt concentration a potential is generated. The Nernst equation can be used to calculate the activity ratios for the cation from the generated potential. The calomel electrodes used should not develop significant tip potentials for the solutions used. (Electrode asymmetry, which was less than 1 mV, was used to correct the measured value). In any event, the same electrodes were used to make the measurements for both the ion exchange membranes and the lipid bilayer membranes.

Once the ion activity ratios were obtained they could be plugged into the Goldman-Hodgkin-Katz equation and used to convert the measured reversal potentials into permeability ratios.

In practice this procedure was only used for the $CaCl_2$ solutions. The salt activity ratios for KCl were assumed to be very close to the single ion activity ratios for K^+ for the solutions used. As a verification of the procedure we calculated salt activity ratios from the single ion activity ratios which we measured with the ion exchange membranes and the resultant values agreed with literature values within 2%.

Results and Discussion

All experiments were performed on planar lipid bilayers made by the procedure of Montal and Mueller [10]. Erythrosine was usually added symmetrically, i.e. to both aqueous phases on each side of the membrane and the permeability of the membrane to ions was monitored by measuring the current in response to an applied transmembrane voltage. (For a detailed description of the circuitry used, see Ref. 11.) In a typical experiment a phospholipid bilayer membrane having a very low ion permeability (conductance of less than 10^{-11} S) was made in the presence of say 0.1 M KCl in the aqueous medium. Erythrosine was added to each side to a final concentration of 40 μM. The conductance of the membrane increased slowly at first to a value of only $3.6 \cdot 10^{-11}$ S ($2.1 \cdot 10^{-7}$ S $\cdot$ cm^{-2}) after 15 min. However, the rate of conductance increase increased with time so that after another 15 min the conductance was now $2 \cdot 10^{-10}$ S ($1.2 \cdot 10^{-6}$ S $\cdot$ cm^{-2}). The slow development of ion permeability and the supralinear rate of rise are reminiscent of the observations of Augustine and Levitan [3] for the action of erythrosine at the neuromuscular junction.

Since erythrosine bears two negative charges at neutral pH, it was necessary to determine whether the observed ion flux was simply due to erythrosine ions flowing across the membrane or to salt ions being carried across the membrane by erythrosine. This was answered by applying either a concentration gradient of KCl or of erythrosine. In the presence of symme-

trical salt solutions (0.1 M KCl) virtually no current flowed across the membrane in the presence of a 5-fold gradient of erythrosine (1.7 mV positive in the high-erythrosine side was required to bring the current to zero). On the other hand, in the presence of a 2-fold gradient of KCl and symmetrical erythrosine a large current was observed in the absence of a trans-membrane voltage difference. (A 13.0 mV potential difference negative in the high KCl side was needed to bring the current to zero when 1.0 M vs. 0.5 KCl was used). Therefore erythrosine induced an increase in permeability of the lipid bilayer to ions and the flow of erythrosine ions across the membrane is small by comparison. From the sign of the potential needed to bring the current to zero it is clear that K^+ flows across the membrane. However, since the ideal Nernst potential was not observed then probably Cl^- is also crossing the membrane.

Fig. 1 illustrates a typical response which was observed when erythrosine was added symmetrically in the presence of a 2-fold gradient of KCl. (In Fig. 1 the potential needed to bring the current to zero was 9 mV. The variability in the value of this potential ranged from 8 to 14 mV in six experiments. This

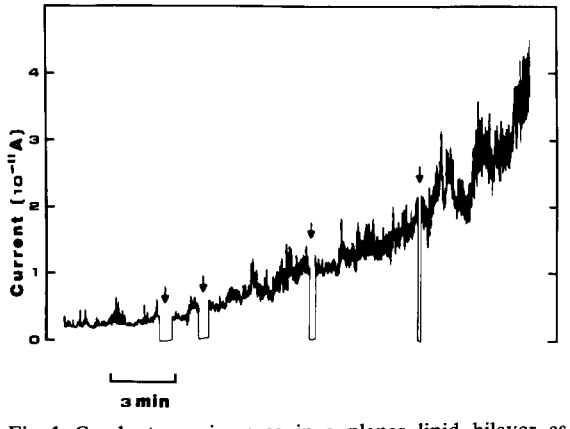

Fig. 1. Conductance increase in a planar lipid bilayer as a result of the addition of erythrosine to the aqueous phase. One side of the membrane faced a solution containing 0.1 M KCl. The opposite side of the membrane faced 0.2 M KCl. The figure is a careful tracing of the original record. During the intervals indicated by the arrows the voltage on the 0.2 M KCl side of the membrane was raised to 9 mV negative as compared to the 0.1 M KCl side. Otherwise the voltage was maintained at zero. The erythrosine B concentration was 80 μM in the aqueous phases on each side of the membrane. The same experiment performed in $CaCl_2$ medium produced a record with much less 'noise'.

variability probably stems from a dependence of the selectivity on membrane dye concentration which is currently under investigation.) The noise in the trace was due to the added erythrosine because the noise in the absence of erythrosine was $\leqslant 10^{-13}$ *A* within the time response of the chart recorder 9 Hz. It did not appear to be due to erythrosine induced membrane instability because membranes lasted for a long time (hours). Therefore the noise is probably due to large fluctuations in ion flow through the membrane due to the assembly and disassembly of permeability pathways. These large fluctuations in permeability indicate that erythrosine, despite its low molecular weight, 880, forms pathways that are highly permeable to ions. Discreet discontinuities in conductance, which might be due to unit pathways, were observed but these were both rare and not of uniform size. This is not unusual and might be expected if the pathway were a dynamic structure undergoing rapid change in its permeability properties.

The permeability induced in the lipid bilayer by erythrosine extends to Ca^{2+}. Table I summarizes results obtained with KCl and $CaCl_2$. Both cations are more permeable than chloride. (The permeability ratio for $CaCl_2$ describes behaviour in the absence of an electric field. The Ca^{2+}/Cl^- ratio would be twice as large in the presence of a field due to calcium's double charge.) That potassium is somewhat more permeable than calcium is seen from Table I and from an experiment in which 100 mM KCl was placed on one side of the membrane and 50 mM $CaCl_2$ on the other. At zero current the KCl side was negative as compared to the $CaCl_2$ side by 6.5 and 5.3 mV in two experiments. (This variation between experiments is probably due to a variation of selectivity with dye concentration in the membrane). Similar experiments with NaCl vs. $CaCl_2$ showed positive potentials in the NaCl side by 7.2 and 9.1 mV indicating that Na^+ is less permeable than Ca^{2+}. Finally, a similar experiment using $MgCl_2$ vs. $CaCl_2$ (both at 0.1 M) yielded a positive potential in the Mg^{2+} side of 8.3 mV indicating a 2-fold selectivity of Ca^{2+} over Mg^{2+}. Because Cl^- is also permeable in erythrosine-treated membranes it would be difficult to obtain quantitative permeability ratios from these experiments. Nevertheless the qualitative effects are clear.

The mechanism by which erythrosine increases the

TABLE I

SELECTIVITY OF THE ERYTHROSINE CONDUCTANCE

Membranes were made as described in the Methods section in the presence of salt solutions with compositions indicated in the table. The activity ratio was calculated from values for salt activity coefficients obtained from the literature. The potential difference shown in the fourth column is the value of the potential that must be applied across the membrane in order to bring the current to zero (net cation flux X cation charge = net anion flux). The value indicated for the potential is a mean (the range is shown in parenthesis). There is strong evidence, not presented in this paper, that the potential difference depends on the conductance of the membrane (hence the variability). The permeability ratio was determined from the potential difference (column 4) by using the constant field approximation (Goldman-Hodgkin-Katz equation). For KCl the salt activity ratios were used in the above calculations but for $CaCl_2$ single ion activity ratios were used. These were measured as described in Methods. Column 6 shows values for potentials measured with the cation exchange membrane and values for the activity ratio for Ca^{2+} in parenthesis.

Salt	Concentration (M)		Activity ratio	Potential difference (mV) (Side 1–Side 2)	Permeability ratio (cation/anion)	Potential difference (mV) (using cation exchange membrane)
	Side 1	Side 2				
KCl	1.0	0.5	1.82	−13 (−11 to −14)	12.5	
	0.2	0.1	1.86	−9 (−8 to −10)	3.7	
$CaCl_2$	0.1	0.05	1.79	−4.8 (3.6 to −5.8)	2.4	−6.8 (1.72)
	0.02	0.01	1.79	−4.7	2.3	−6.9 (1.73)
	0.03	0.01	2.59	−7.1 (−6.5, −7.6)	2.3	−10.8 (2.36)

membrane's permeability to ions has proven to be quite complex. The presence of cooperativity was evident from the supralinearity of the time dependent conductance increase demonstrated in Fig. 1. Experiments were done to determine how the conductance induced in the lipid bilayer varies as a function of erythrosine concentration. Unfortunately. under most conditions examined to date, rather than obtaining a steady-state conductance when an aliquot of erythrosine was added to the chamber one obtained a steady increase in conductance. (The supralinearity in the time course was limited to the early phase of the first dye addition. At later times the rate of conductance increase was constant and proportional to the medium dye concentration.) Therefore the results shown in Fig. 2 depict the rate of conductance increase as a function of erythrosine concentration. The slope of this function increases from 4.5 to 6.6 from low to high conductances. Thus the conducting units are large complexes composed of many monomers.

By contrast the dependence on Ca^{2+} concentration is linear. A neutral membrane, composed of diphytanoylphosphatidylcholine, made conductive by the addition of erythrosine in the presence of 2 mM $CaCl_2$ did not instantaneously increase its conductance when more Ca^{2+} was added to the medium. There was a slow increase in conductance whose final level was dependent on the Ca^{2+} concentration in a linear fashion. Therefore it appears that at this concentration of Ca^{2+} the conducting pathways are operating at maximum rate and the addition of more Ca^{2+} causes more pathways to be assembled.

All the experiments described thus far were performed at low potential differences across the membrane ($\leqslant$25 mV). If higher fields are used erythrosine displays a remarkable increase in its conductance with increasing voltage. Fig. 3 shows that the conductance increases exponentially for 22 mV. Although this is a rather weak dependence as voltage-dependent conductances go, it occurs over a large voltage range thus producing an enormous increase in conductance. In the voltage range which we explored ($<$200 mV) we saw no tendency toward saturation.

Our findings with erythrosine are generally consistent with the results reported by Augustine and Levitan [3] and Levitan [13] on neurons. One major difference is the high power dependence of the conductance on the erythrosine concentration which we observe. The above investigators find that their effects are dependent on the dye concentration to the 0.7 power [4]. Since they were monitoring trans-

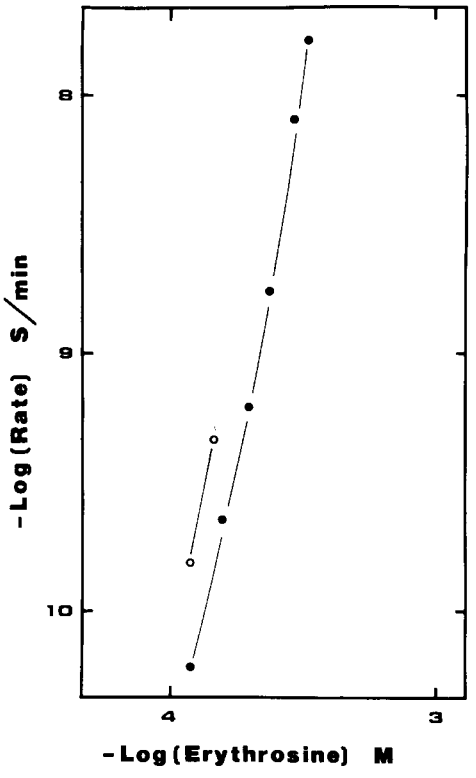

Fig. 2. The variation of membrane conductance with erythrosine concentration. Membranes were made in the presence of 80 mM $CaCl_2$. The open symbols represent data gathered on one membrane while the closed symbols represent a second membrane. Erythrosine B was added symmetrically and sufficient time was allowed to pass such that the initial supralinearity became a constant rate of conductance increase. Once a steady rate was achieved more erythrosine was added symmetrically and a new rate was obtained. The process was repeated until the membrane broke. The rate of increase is expressed in $S \cdot min^{-1}$. For the experiment designated by the filled symbols the membrane conductances just before the the next dye addition were $1.0 \cdot 10^{-9}$ S, $2.7 \cdot 10^{-9}$ S, $1.1 \cdot 1.0^{-8}$ S, $2.4 \cdot 10^{-8}$ S, $8.2 \cdot 10^{-8}$ S and $1.6 \cdot 10^{-7}$ S.

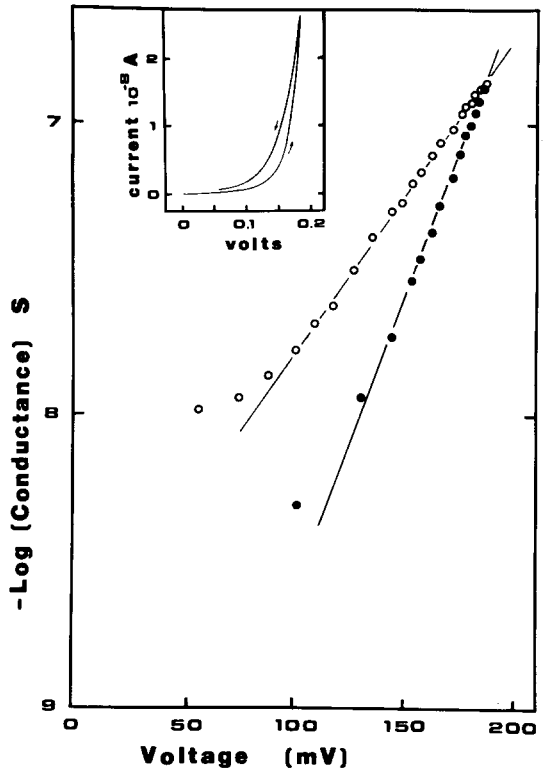

Fig. 3. The voltage dependence of erythrosine B's conductance. The membrane was made using diphytanoylphosphatidylcholine instead of soybean phospholipids. The erythrosine concentration was 400 μM in both aqueous phases which consisted of 2 mM $CaCl_2$. (Similar results were obtained with charged membranes and at higher $CaCl_2$ concentrations). The voltage was varied by means of a triangular voltage wave with a frequency of 5 mHz. The inset shows a tracing of the experimental curves. As indicated by the arrows, the lower curve was generated with constantly increasing voltage while the upper curve was generated with constantly decreasing voltage. The chord conductance was calculated for a variety of points along these curves and plotted as a log function in the main figure. The filled circles represent points from the lower curve in the inset while the open circles designate values for the upper curve in the inset.

mitter release as depolarizations (m.e.p.p.'s) at the postsynaptic terminal, it is likely that events between the release of Ca^{2+} into the cytoplasm and transmitter release were altering the dose-response curve. Possible factors include: calcium sequestering systems; the voltage dependence of erythrosine action coupled with its effect on transmembrane voltages; the dependence of transmitter release on intracellular Ca^{2+} concentrations; the dependence of cytoplasmic free calcium concentration on the rate of erythrosine-induced calcium flux into the cytoplasmic space.

The observations described above show that erythrosine increases the permeability of a lipid bilayer to a number of ions including Ca^{2+}. Membrane proteins are not required for this function. Since most biological membranes have areas of lipid bilayer, erythrosine should be able to enter these areas and increase the membrane's permeability to Ca^{2+} and other ions. Also the fact that erythrosine is a small molecule

which partitions well into octanol (The octanol: water partition coefficient was reported [3] to be 0.71.) would indicate that it could enter most biological membranes. It should also be able to cross the membranes and act on the subcellular organelles. The voltage dependence of the permeability induced by erythrosine means that membranes, such as the inner mitochondrial membrane, which have large potentials across them will become 10- to 100-times more permeable to Ca^{2+}, at equal doses of dye, than membranes without an electric field across them. If mitochondria store Ca^{2+} [17], erythrosine might release it thus elevating cytoplasmic levels.

Normally mammalian cells have a large Ca^{2+} concentration gradient across their plasma membrane (2 to 3 mM extracellular and $\simeq 0.1$ μM intracellular). Erythrosine should then act on all these cells to elevate internal Ca^{2+} which in turn should perform a myriad of functions depending on the cell type. The degree of effect of erythrosine will depend on the relative magnitude of the erythrosine induced permeability and the ability of the cellular systems to maintain normal intracellular levels of Ca^{2+}.

Are the fluxes of Ca^{2+} which we observed on the planar bilayers sufficient to increase intracellular Ca^{2+} levels in view of the high sequestering capacity of cytoplasm and cytoplasmic organelles? We shall attempt to answer this question by using results obtained by Rose and Loewenstein [14] for microinjections of Ca^{2+} into salivary gland cells. Assuming that all the iontophoretic current they applied was due to Ca^{2+}, they found the injection of $5 \cdot 10^{10}$ calcium ions swamped the sequestering system of their 100 μm diameter cells for 4 or 5 s. In our experiments, the presence of 100 μM erythrosine could increase the conductance of a lipid bilayer to 10^{-8} S in 30 min (in the presence of 2.0 mM $CaCl_2$ in the medium). This conductance allows a net flow of $5 \cdot 10^9$ calcium ions per s through an area of membrane comparable to the surface area of the salivary gland cell. (This assumes a driving force of 100 mV. Across surface membranes this driving force may only be 70 mV but in addition there is a 10^4 chemical gradient.) Clearly the steady flow of $5 \cdot 10^9$ calcium ions per s into this cell will change its free level of Ca^{2+}. (Longer exposure to erythrosine B will increase Ca^{2+} flux further.) If cells with smaller diameters are chosen, the increase in surface to volume ratio will favor the dye over the sequestering system. Therefore the high surface to volume ratio of nerve terminals might make them particularly susceptible. Clearly these arguments apply mainly to transient exposures to erythrosine.

The effects of long term exposure to erythrosine must be countered by the Ca^{2+} pumps of the plasma membrane. The ability of cells to extrude calcium ions varies from cell to cell but is usually <1 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ [15,16]. In the phospholipid bilayer membranes used in these studies, a conductance of 10^{-8} S is equivalent to a net flux of 50 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ (assuming a 100 mV driving force).

Acknowledgement

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