ELECTROIMMUNOASSAY

A New Competitive Protein-binding Assay Using Antibody-sensitive Electrodes

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ABSTRACT A novel technique is reported in which an antibody-sensitive electrode for anti-prostaglandin E_2 antisera was used to measure solution-phase PGE_2 in nanomolar quantities. The electrode was constructed by incorporating a cation-selective ionophore-hapten (PGE_2) conjugate into a polyvinyl chloride membrane. Transmembrane potential in a fixed potassium gradient was measured. The addition of anti-PGE_2 antisera changed membrane potential in a concentration-dependent manner. The effect of anti-PGE_2 antibodies on membrane potential was decreased by adding free PGE_2 to the buffer-containing antisera. With this technique a competitive protein-binding assay was developed, and standard curves for solution-phase PGE_2 were generated over a concentration range of 1 to 1,000 nM. The assay was relatively specific for PGE_2; PGD_2 and PGF_{2a} had only minor effects on transmembrane potential over the effective concentration range for PGE_2.

INTRODUCTION

Radioimmunoassay (RIA) caused a revolution in the technology available to endocrinologists and clinical chemists, because it allowed relatively quick and inexpensive analyses of biological chemicals that occur naturally in small quantities. The few drawbacks of RIA are (*a*) the radioisotopes used are expensive and require special handling, and (*b*) the turn-around time for a measurement can last several hours to several days if the expense of the assay requires that many samples be accumulated before it becomes economical to run an assay. Three years ago Solsky and Rechnitz (1979) reported production of an electrode capable of measuring antibody concentration. This was achieved by incorporation of an ionophore-hapten conjugate into a polyvinyl-chloride membrane. Antibodies raised against the hapten used in the conjugate were specifically sensed by the membrane causing an apparent shift in the ion selectivity of the membrane (Solsky and Rechnitz, 1981). Based on this previous work, it was likely that other antibody-sensitive electrodes could be constructed for other antibody-hapten systems. We reasoned that if the antibody-hapten interaction at the surface of antibody-sensitive membranes were reversible, then it would be possible to produce competition between the membrane-conjugated hapten and hapten free in solution. If the solution-phase hapten was present in high enough concentration, most of the antibody molecules would be unavailable to affect membrane potential. This should produce potential changes across the membrane equivalent to reducing antibody concentration. These are the conditions of a competitive protein binding assay where the dependent variable is a potentiometric measurement. The relative speed, simplicity, and freedom from use of isotopes and hazardous chemicals make such a technique an attractive alternative to radioimmunoassays.

We have tested this hypothesis by constructing plastic membranes containing covalent conjugates of dibenzo-18-crown-6 (a cation-selective ionophore) and PGE_2. The membranes were installed in electrodes that responded with potential changes to antibodies raised against PGE_2-protein conjugates. We have used these antibody-sensitive electrodes in an electroimmunoassay, a competitive protein-binding assay that quantitates solution-phase PGE_2 concentration by measuring transmembrane potential.

METHODS

Chemistry: Synthesis of Ionophore-Hapten Conjugate

Dibenzo-18-crown-6 was selected as the ionophore because its structure is ideal for chemical modification. The benzene rings were used to attach new functional groups to the ionophore to facilitate covalent coupling of PGE_2. An amide linkage utilizing the carboxylic group of PGE_2 was chosen for conjugation to the ionophore, because this was the site where PGE_2 was coupled to bovine thyroglobulin to produce antibodies directed against PGE_2 (Sanders, 1976). Dibenzo-18-crown-6 was nitrated; the dinitro compound was reduced to a diamine; and an amide linkage to PGE_2 was produced by the mixed anhydride method (Bodanszky and Ondetti, 1966).

Synthesis of the *trans*-diamine of dibenzo-18-crown-6 was carried out in three steps: (*a*) nitration of dibenzo-18-crown-6, (*b*) separation of the *cis* and *trans* dinitro products, and (*c*) reduction to the *trans*-diamine. The nitrated crown ether was produced by refluxing dibenzo-18-crown-6 (PCR Research Chemicals Inc., PCR Inc., Gainsville, FL) with nitric and acetic acid. *Cis* and *trans* dinitro products were separated by crystallization. The *trans*-diamine was prepared by the catalytic reduction of the *trans* dinitro product. The crude diamino product was purified by sublimation, and the synthesis was verified by melting point (MP) (product, 198–203°C; literature, 199–203°C) and by infrared (IR) (Feigenbaum and Michel, 1971).

PGE_2 was reacted with triethyl amine and ethyl chloroformate to produce a mixed anhydride of prostaglandin. Diamino-dibenzo-18-crown-6 was added to the PGE_2-mixed anhydride to covalently couple prostaglandin to the crown ether via amide linkages. This resulted in the formation of the PGE_2-*trans*-diamide of dibenzo-18-crown-6 (MP 134°C). The product was purified by recrystallization in chloroform and ethanol, and verified by IR, nuclear magnetic resonance (NMR), and fast atom bombardment mass spectral analysis.

Production and Testing of Antibody-sensitive Electrode

Polyvinyl chloride (PVC) membranes were prepared by dissolving ionophore or ionophore-PGE_2 conjugate (1 mg/250 mg PVC) PVC, and dibutyl sebacate in tetrahydrofuran. The solvent was removed by slow evaporation, resulting in the formation of a flexible membrane, 0.2 mm thick and 50 mm in diameter. A disk of the plastic membrane (4 mm x 0.2 mm) was installed into an electrode housing (92-00; Orion Research Inc., Cambridge, MA), which was positioned in a 4-ml jacketed Lucite test chamber. Temperature in the internal compartment of the test chamber was maintained at 25°C by temperature regulated water flowing through the outer compartment. The solution in the test chamber was stirred with an air-driven magnetic stirring device and a small Teflon stir bar. The solution could be exchanged via input and output ports in the test chamber. Transmembrane potential was measured with an electrometer (701; Orion Research Inc.) coupled to a junction reference electrode (90-02; Orion Research Inc.). The internal chamber of the Orion 92-00 electrode was filled with 0.01 M KCl and was soaked in 0.1 M KCl for several hours prior to testing. The external buffer solution (EBS) consisted of 0.001 M KCl, 0.001 M Tris, and 0.051 M $CaCl_2$, such that the ionic strength and pH were constant in all solutions at 0.154 M and pH 7.2, respectively. Calcium and Tris ions had little or no effect on membrane potential in the concentrations used. Data were recorded either manually from the digital readout of the electrometer, or by a strip chart recorder (2200; Gould Inc., Instruments Div., Santa Clara, CA).

RESULTS

Membrane Response to Anti-PGE_2 Antiserum

Antibodies against PGE_2 were raised in New Zealand white rabbits by immunizing with covalent conjugates of bovine thyroglobulin and PGE_2 (Sanders, 1976). The sera of preimmunized and immunized rabbits were collected, lyophilized, and reconstituted in EBS. The effects of these sera on membrane potential were tested on membranes containing conjugates of dibenzo-18-crown-6 and PGE_2. The reconstituted sera were added to the test chamber in 20-μl aliquots. After each addition, the solution was stirred and the steady state potential was recorded. After a total addition of 100 μl, the membrane was washed by drawing fresh buffer (50 ml) across the surface by suction. Then the test chamber was emptied and refilled with fresh buffer.

The nonimmune serum had little effect on transmembrane potential, but sera from immunized animals produced a concentration-dependent shift in membrane potential (Fig. 1). The change in potential was similar to that caused by increasing the K^+ concentration of the external buffer, suggesting that the anti-PGE_2 antibodies either affected the potassium gradient near the membrane surface or decreased potassium selectivity. Antisera preabsorbed to saturation with PGE_2 had negligible effects on membrane potential. The antibody-membrane interaction was also tested on membranes containing dibenzo-18-crown-6 in a similar concentration as above, but without PGE_2 covalently coupled. These membranes were unresponsive to either the immune or the nonimmune sera, supporting the concept that the interaction between membranes containing ionophore-hapten conjugates and antibodies to the hapten is depended on the antibody-hapten affinity.

The effect of the antibody on membrane potential was reversible. The average time constant of the potential response to antibody was 7.2 ± 0.3 min. When the electrode was returned to buffer, the pre-antibody membrane potential was restored with an average time constant of 3.6 ± 0.2 min.

Effect of Solution-Phase PGE_2 on the Antibody Responses

The reversibility of the interaction between antibodies and PGE_2 conjugated to ionophore suggested that competition between membrane and solution-phase PGE_2 molecules might be exploited in a competitive protein-binding assay in which membrane potential is the dependent variable (Fig. 2). To test this hypothesis anti-PGE_2 serum was diluted in EBS at a concentration from the linear portion of the antibody-response curve (Fig. 1). Aliquots of the

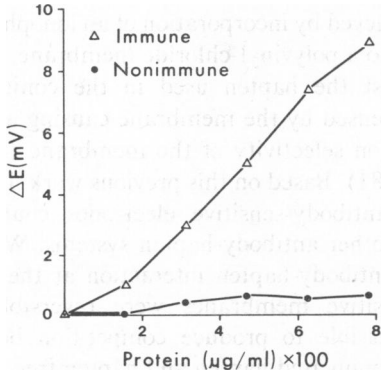

FIGURE 1 Response of antibody-sensitive PVC membranes to immune, anti-PGE_2 (△), and nonimmune (●), sera. Membrane potential was changed in a concentration-dependent manner by immune serum added to the external solution. Concentration of antibody is expressed as total protein (Lowrey et al., 1951).

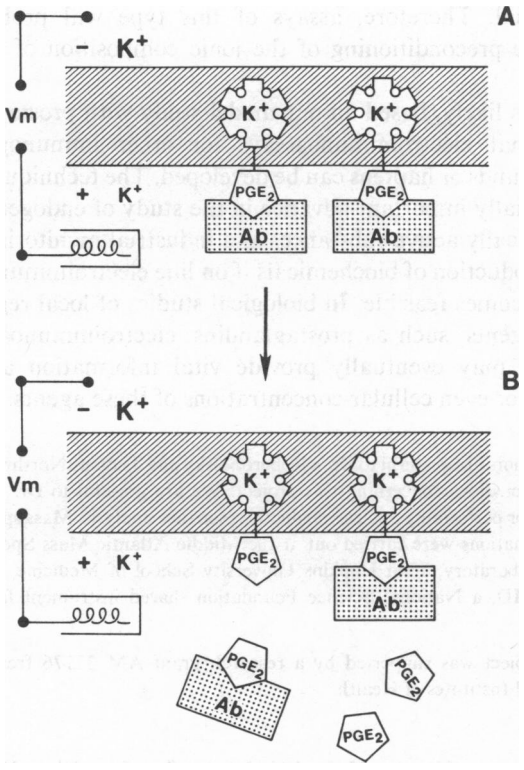

FIGURE 2 Schematic representation of electroimmunoassay. *A* shows a hypothetical interaction between fixed PGE_2 molecules conjugated to the ionophore and anti-PGE_2 antibodies. This interaction changes the transmembrane potential (Vm). The addition of free PGE_2 to the system results in competition between solution-phase and membrane-phase PGE_2 for antibody binding sites (*B*). As the concentration of free PGE_2 is increased, a greater percentage of antibody molecules are displaced from the membrane, resulting in a reduction in the voltage response caused by the antibody.

diluted serum were added to a series of tubes. PGE_2 was added to each of the tubes to achieve concentrations ranging from 3 to 860 nM. The electrode was first immersed in an antiserum solution containing no PGE_2, and the maximal voltage response (V_o) was recorded. Then the membrane was washed with buffer and the electrode was immersed in one of the antiserum solutions containing PGE_2. This cycle was repeated until potential measurements had been made on each of the PGE_2-containing antiserum samples. The V_o response of the membrane was reduced in a concentration-dependent manner by solution-phase PGE_2. These data provide a standard curve, such as that shown in Fig. 3, which can be used for an electroimmunoassay. The response time constants for samples containing PGE_2 were similar to the time constants of samples without PGE_2, so an entire standard curve could be generated in <1 h.

Specificity of the PGE_2 Assay

An important feature of a competitive protein-binding assay is the series of tests in which other prostaglandins of similar structure (PGD_2 or PGF_{2a}) were substituted for

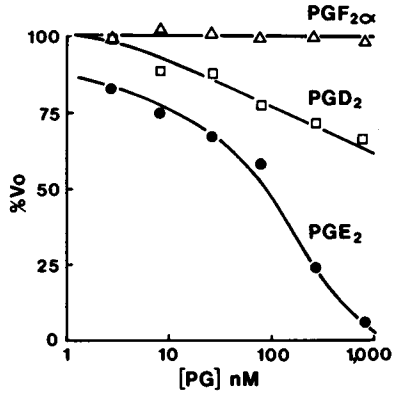

FIGURE 3 Standard curves for electroimmunoassay. A concentration of antibody in the linear portion of the antibody-response curve (Fig. 1) was selected. The voltage response at this antibody concentration (V_o) was decreased as a function of the solution-phase concentrations of PGE_2 and PGD_2, but not by PGF_{2a}.

PGE_2 to generate standard curves as described above. PGF_{2a}, a compound in which the keto function on the cyclopentane ring of PGE_2 is replaced with a hydroxyl group, had negligible effects on the membrane response to the antibody. PGD_2, in which the keto and hydroxyl functions of the cyclopentane ring are reversed, demonstrated limited ability to reduce the effect of the antibody. The specificity of the antibody in the electroimmunoassay was similar to the specificity of the antibody in standard radiometric assays (Sanders, 1976). Fig. 3 shows a comparison of standard curves in which PGD_2 and PGF_{2a} were substituted for PGE_2.

DISCUSSION

The major new concept introduced in this study is that antibody-sensitive electrodes (Solsky and Rechnitz, 1979) can be used in a competitive, protein-binding assay to measure solution-phase hapten concentration. The specificity of this electroimmunoassay was similar to that demonstrated by the antibody in standard radiometric assays, but the sensitivity was lower (1–1,000 nM as compared with 0.1–100 nM in a radiometric assay; Sanders, 1976). It is conceivable that the performance could be considerably enhanced by monoclonal antibodies (greater specificity) or certain physical changes, such as membrane geometry (surface area or thickness), conjugate concentration, or alterations in the covalent binding of the hapten to the ionophore (insertion of spacer groups between the ionophore and the hapten or increasing the hapten to ionophore binding ratio).

There are several reasons to assume that the effect of the anti-PGE_2 antiserum on membrane potential was a specific antibody-hapten interaction. Membrane potential was affected by serum from immunized animals, but not by serum from nonimmunized animals. This suggests that the effect was not a nonspecific effect of protein on the membrane. The effectiveness of the anti-PGE_2 antiserum

to alter membrane potential was abolished by preabsorbing the antibody with PGE_2, but not by similar concentrations of PGF_{2a}. The similarities in the structures of PGE_2 and PGF_{2a} suggest that there is highly specific recognition of the membrane-bound PGE_2 by the antibody. Membranes containing ionophore that was not conjugated with PGE_2 were not responsive to either immune or nonimmune sera.

The mechanism for the specific effect of antibody on membrane potential is unknown. Solsky and Rechnitz (1981) suggested that the antibody interaction may have increased the selectivity of the membrane. Our results suggest a different mechanism because the presence of antibody caused a shift in transmembrane potential that is more consistent with a decrease in selectivity or a decrease in the potassium gradient. The latter might be due to the acidic properties of serum proteins at pH 7.2 (Harper et al., 1979), which might bind potassium and increase the local concentration at the membrane surface. The mechanism of the solution-phase hapten on the antibody-membrane effect, while not directly studied in our experiments, is consistent with the concept depicted in Fig. 2. It is likely that the free PGE_2 competes with the membrane-bound PGE_2 for antibody binding sites. At high concentrations of solution-phase PGE_2 most of the antibody binding sites are occupied and unavailable to interact with the membrane. This would have the same effect on membrane potential as would lowering the antibody concentration.

A major drawback to electroimmunoassay as presented here is the dependence on the ionic gradient, ionic strength, and pH. In fact a criticism of our findings might be that we changed the ionic concentration in our EBS by adding reconstituted serum that was not dialyzed. We controlled the addition of contaminant ions by performing tests in which buffer solutions containing the ions at concentrations found in sera were added in volumes equal to the antibody additions. These tests showed that the low concentrations of contaminant ions introduced in our serum dilutions either had no effect on membrane potential or a slight change (<1 mV) in the opposite direction from the effect of the antibody containing sera. Higher concentrations or extended exposure to contaminant ions could conceivably produce artifactual changes in membrane potential. Therefore, assays of this type will probably require preconditioning of the ionic composition of samples.

It is likely, based on our model study with prostaglandin, that electroimmunoassays for other immunogenic compounds or haptens can be developed. The technique is a potentially important advance in the study of endogenous, biologically active substances and industrial monitoring of the production of biochemicals if on line electroimmunoassay becomes feasible. In biological studies of local regulatory agents, such as prostaglandins, electroimmunoassay probes may eventually provide vital information about tissue, or even cellular concentrations of these agents.

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