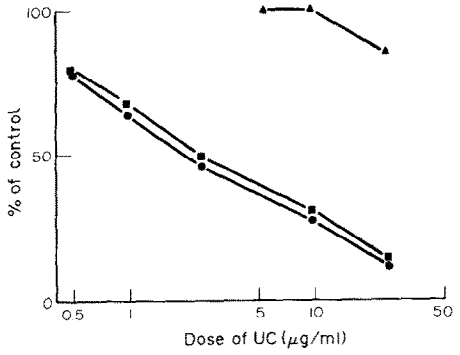

Fig. 3. Effect of UC on macromolecular synthesis. Exponentially growing cells were treated with UC for 2 hr in a medium containing [^{3}H]-labelled precursor (dThd, Urd, or Leu). Incorporation of labelled precursor into cells was measured. Incorporation ratio (%) relative to control cells is shown. DNA (●), RNA (■), and protein (▲) syntheses.

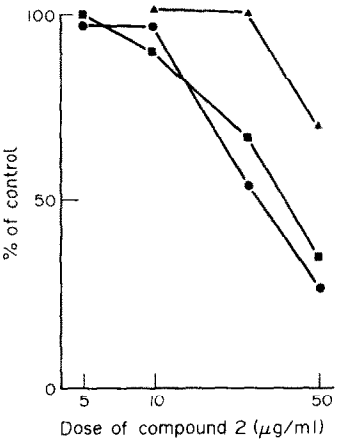

Fig. 4. Effect of compound 2 on macromolecular synthesis. See the legend of Fig. 3.

syntheses. This differs from ulithiacyclamide, a UC analogue, which is a potent cytotoxic agent that has an inhibitory effect on protein synthesis.

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Identification of multiple G_i subtypes and a novel G protein in bovine kidney cortex

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Heterotrimeric guanine nucleotide-binding proteins (G proteins*) comprise a specific family that serves as intermediaries in a variety of transmembrane signaling processes in eukaryotic cells [1, 2]. Pertussis toxin catalyzes ADP-ribosylation of certain G_α subunits, thereby uncoupling the G protein substrate from its corresponding receptor [1, 2]. Molecular cloning of cDNAs and genes encoding α subunits indicates the existence of at least four genes for the α subunits ($G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{o\alpha}$) of the G proteins serving as the substrate for pertussis toxin, besides two transducin

* Abbreviations: G protein, guanine nucleotide-binding protein; G_i, a G protein originally identified in terms of inhibition of adenylate cyclase; G_o, a G protein of unclear function, abundant in brain; G_x, α subunit of G protein; $G_{x(z)}$, a putative G protein encoded by cDNAs cloned from rat (G_x) and human (G_z) neural libraries; G_s, G protein involved in hormonal stimulation of adenylate cyclase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and DTT, dithiothreitol.

α subunits [3]. Moreover, recently a cDNA encoding a novel, possibly pertussis toxin-insensitive, G_α termed $G_{x(z)}$ was cloned [4, 5].

The multiplicity of G protein subtypes raises important questions regarding tissue distribution of individual subtypes and specific function. Specific mRNA analysis indicates that certain G proteins (e.g. G_{i2}) appear to be ubiquitously expressed, whereas others (e.g. G_o) may be more restricted in expression [4–7]. To study the pattern of G_α expression at the protein, rather than mRNA level, we have raised antisera against synthetic peptides corresponding to sequences uniquely predicted by G_α cDNAs.

Using these antisera, we have now characterized the pattern of G protein expression in kidney cortex. Numerous studies [8–16] suggest that G proteins, both pertussis toxin-sensitive and -insensitive, are involved in signal transduction in renal tissue and kidney-derived cell lines. The present data indicate that kidney cortex contains all three G subtypes, as well as the novel G protein, $G_{x(z)}$.

Materials and methods

Brain membranes were prepared as described earlier [17]. For G protein purification, four bovine kidneys were freshly obtained from a local slaughterhouse, and the cortex was collected immediately. The minced cortex was homogenized in a Waring blender for 1 min with 3 vol. of 10 mM Tris–HCl, pH 7.5, containing 10% sucrose, 5 mM EDTA, 1 mM DTT and 50 KIU (kallikrein inhibitory unit)/ml of aprotinin. The homogenate was combined with an equal volume of the same buffer and filtered through one layer of cheesecloth. Membranes were collected by centrifugation at 12,000 *g* for 60 min at 4°. The membrane pellet was washed and extracted with 1% cholate, and subjected to the following procedures for the purification of G proteins [18]. Protein determinations were made with the Bio-Rad protein assay and by staining with Amido Black [18].

The procedures employed to purify pertussis toxin substrates are based on previous methods with slight modification [18]. Three successive chromatography steps with DEAE-Sephacel, Sephacryl S-300 HR and DEAE-Toyopearl 650(S) resulted in the separation of two major peaks (peaks I and II) of the pertussis toxin substrate and GTPγS-binding activity, and a small peak of GTPγS-binding activity without detectable [^{32}P]ADP-ribosylation substrate (see Fig. 1A). The polypeptide composition of the fractions was analyzed by SDS–PAGE, autoradiography and immunoblotting.

The pertussis toxin substrates were identified by their ability to be [^{32}P]ADP-ribosylated in the presence of pertussis toxin and [^{32}P]NAD, and by their ability to bind [^{35}S]GTPγS as in Ref. 18. SDS–PAGE and immunoblot analysis were performed essentially as in Ref. 19. Antibodies used in this study were defined previously [20], except for antiserum QN which was raised against the carboxy-terminal decapeptide of the α subunit of $G_{x(z)}$. Detailed characterization of this antiserum which showed no detectable cross-reactivity with any other known G protein will be presented elsewhere.*

Results and discussion

Pertussis toxin-catalyzed ADP-ribosylation and one-dimensional SDS–PAGE cannot discriminate several structurally, and possibly functionally, distinct G proteins [19]. Therefore, we applied an immunoblot technique with specific antibodies against synthetic decapeptides to discriminate G proteins of similar molecular size.

To characterize the apparent heterogencity of G protein expression in renal cortex, we attempted to isolate G proteins from bovine kidney cortex membranes. Soluble proteins extracted from membranes with 1% sodium cholate were successively applied to columns of DEAE-Sephacel, Sephacryl S-300 HR and DEAE-Toyopearl 650(S). Following these three chromatographic steps, we detected two peaks containing both pertussis toxin substrate and GTPγS-binding activity (Fig. 1A). The polypeptide compositions of the two peaks analyzed by SDS–PAGE with Coomassie Blue staining and autoradiography after pertussis toxin-catalyzed ADP-ribosylation are shown in Fig. 1, B and C, respectively. There were three polypeptide bands migrating at about 40–41 kD in the Coomassie Blue-stained gel. Only two bands, however, were detected by autoradiography. This suggests that one of the bands detected by protein staining may not serve as a substrate for pertussis toxin.

Immunoblotting experiments were carried out to identify the pertussis toxin substrates contained in the two peaks (Fig. 2). Briefly, antiserum AS recognizes $G_{i\alpha}$ ($G_{i1\alpha} = G_{i2\alpha} \gg G_{i3\alpha}$) but not $G_{o\alpha}$; antiserum LD recognizes a $G_{i1\alpha}$ specific peptide sequence; antiserum LE recognizes a $G_{i2\alpha}$ specific peptide sequence; antiserum SQ reacts with a $G_{i3\alpha}$ specific peptide sequence; antiserum GO reacts with carboxy-terminal sequence of $G_{o\alpha}$ [19, 20, 22]; QN antiserum, developed to react with a $G_{x(z)}$ carboxy-terminal peptide sequence, has no cross-reactivity with $G_{i\alpha}$, $G_{o\alpha}$ or $G_{s\alpha}$. Antiserum AS detected immunoreactivities of two bands (41 kD and 40 kD) in both fractions. Antiserum LD reacted with a single 41 kD peptide in both fractions. Antiserum LE reacted with a single 40 kD peptide in both fractions. Antiserum SQ showed reactivity in a single 41 kD peptide in both fractions. Antiserum QN reacted with a single peptide in both fractions (Fig. 2, A and B). Antiserum GO strongly reacted with a 39 kD band in a rat brain cholate extract used as a positive control, but failed to react with a band of similar migration in either fraction of renal G proteins (data not shown). Moreover, immunoreactivity of fraction 22 against QN antiserum was examined. QN immunoreactivity in fraction 22 was similar to that in peak II (data not shown). This may indicate that at least part of the GTPγS-binding activity in fraction 22 reflects $G_{x(z)}$, and that since no [^{32}P]ADP-ribosylation substrate was detected in this fraction, $G_{x(z)}$ is not a pertussis toxin substrate. However, we cannot exclude contamination of $G_{x(z)}$ with other pertussis toxin-insensitive G proteins (e.g. small molecular weight G proteins, and G_s) in this fraction.

Characterization of the G_i subtype-specific antisera using purified G proteins [20], and individual G_α subunits expressed in *Escherichia coli**, indicates that the immunoreactivity detected with these reagents, in fact, represents distinct forms of G_i, rather than cross-reactivity. We also detected an approximately 40 kD protein, specifically reactive with antiserum QN, in the G protein fraction partially purified from renal cortex, but not in crude renal cortical or medullary membranes (data not shown). This protein, presumptively $G_{x(z)}$, may be present at too low a concentration to be detectable in crude renal membranes, but is detected upon enrichment of G proteins through partial purification of a cholate extract of renal cortical membranes. In contrast, G_o was undetectable even in the partially purified G protein fraction. Using a different peptide antibody specific for $G_{x(z)}$, Spicher *et al.* [21] also reported detection of $G_{x(z)}$ in kidney, after completion of our study.

mRNA analysis suggests that G_{i2} and G_{i3} are expressed ubiquitously [6, 7]. Up until now, however, G_{i3} had been explicitly identified at the protein level only in HL-60 and red blood cells [22, 23]. Although one study of mRNA distribution [6] suggested that G_{i1} mRNA is widely expressed, the protein itself has been identified only in brain, where it is second in abundance only to G_o [20]. $G_{x(z)}$ protein has not been definitively identified. mRNA analysis suggests expression primarily in neural cells [4, 5]. The present results indicate that small amounts of $G_{x(z)}$, or of a closely-related protein, are present in kidney. Failure to detect G_o may indicate that expression of this protein is truly restricted to neuronal cell types [7].

* Goldsmith P and Spiegel A, manuscript in preparation.

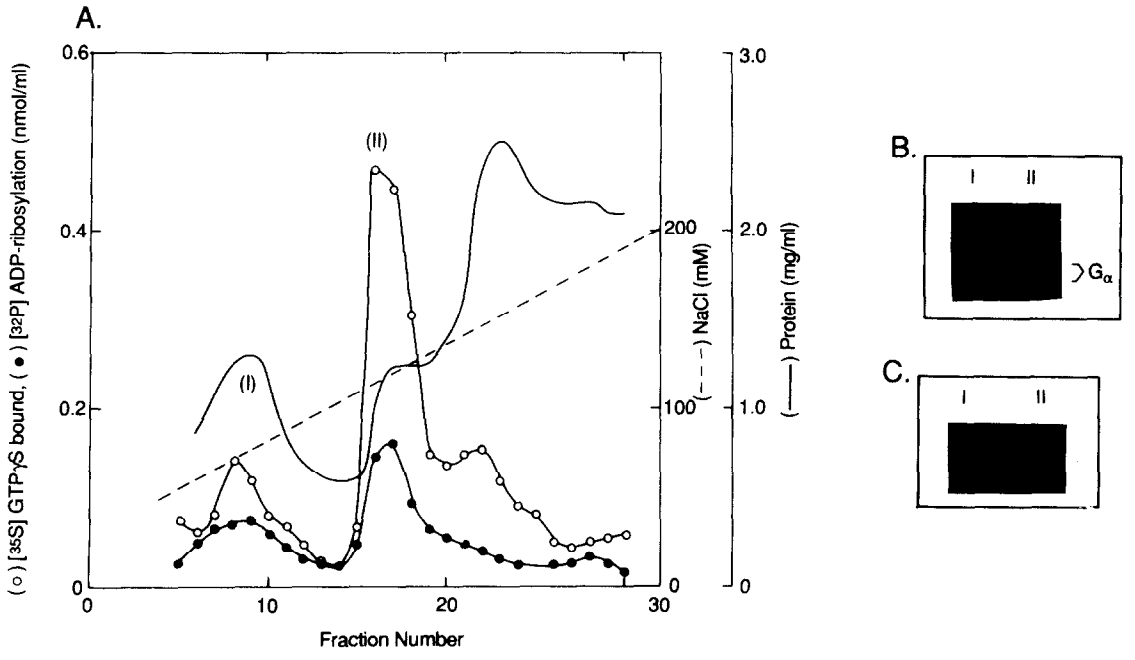

Fig. 1. DEAE-Toyopearl 650(S) chromatography of G proteins. (A) GTPγS-binding activity and pertussis toxin substrate-rich fractions through Sephacryl S-300 HR were applied to DEAE-Toyopearl 650(S), and eluted with a 150-ml linear concentration gradient of NaCl (50–200 mM); 5-ml fractions were collected. G protein activity was quantitated by measuring the [^{35}S]GTPγS-binding activity of each fraction and also the maximal incorporation of [^{32}P]ADP-ribose in each fraction. Aliquots corresponding to peaks I and II were subjected to (B) SDS–PAGE on a 12.5% gel and staining with Coomassie Brilliant Blue, and (C) pertussis toxin-catalyzed ADP-ribosylation and autoradiography. All the procedures and assay conditions used in these experiments were described earlier [18].

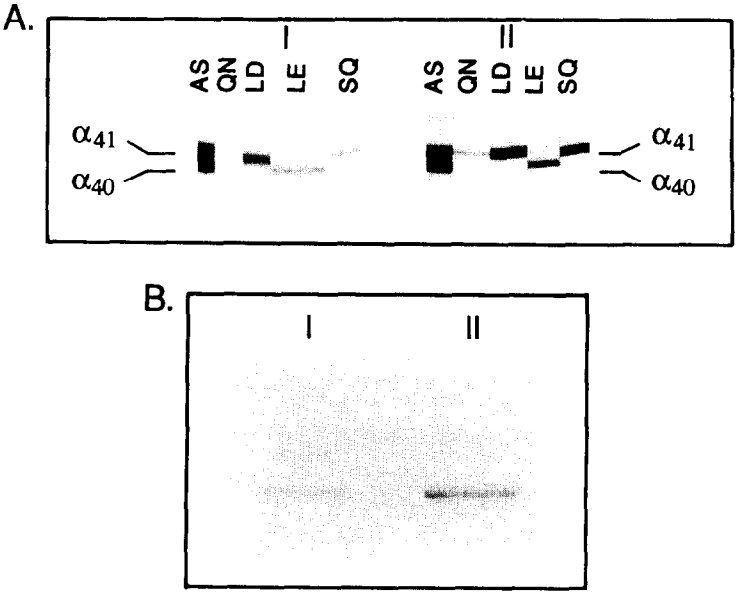

Fig. 2. Reactivity of AS, QN, LD, LE, and SQ antisera with purified fractions. Polypeptide compositions in purified fractions were analyzed by SDS–PAGE on a 12.5% gel, and immunoblotted with antisera as follows; (A) AS, 2 μg/ml; QN, 2 μg/ml; LD, 2 μg/ml; LE, 5 μg/ml; and SQ, 10 μg/ml. (I) The peak of first minor fraction; (II) The peak of the second major fraction. α_{41} and α_{40}, α subunits of 41 and 40 kD, respectively, on SDS–PAGE. (B) QN, 10 μg/ml.

The specific functions, in terms of receptor–effector coupling, of the proteins we have identified in renal tissue are unknown. G protein-coupled receptors for diverse agonists, including prostaglandins, catecholamines, vasopressin, and bradykinin, have been detected in kidney and kidney-derived cell lines [8–16]. Pertussis toxin-sensitive inhibition of adenylate cyclase, and pertussis toxin-insensitive modulation of phosphoinositide metabolism [8–16] are among the G protein-regulated effector activities detected in renal tissue. The present results help to define the specific G proteins which may mediate these functions in kidney. The specific functions of the G proteins we have identified, and whether these proteins are functionally distinct in receptor–effector coupling, must await the results of further studies.

In summary, G proteins were partially purified from bovine kidney cortex membranes and characterized with specific antibodies. The major peak of GTPγS-binding activity contained immunoreactive proteins corresponding to all three G_i subtypes, as well as the novel G protein, $G_{x(z)}$.

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