Immuchemical Characterization and Developmental Expression of Shaker Potassium Channels from the Nervous System of Drosophila

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We have raised antisera against recombinant peptides expressed from cDNAs fragments common to all splicing variants generated at the Shaker locus of Drosophila and used them as a tool to biochemically characterize these channel proteins. This antisera succeeded in detecting the expression of multiple Shaker potassium channels (ShKch), proteins with variable molecular mass (65–85 kDa) and pl (5.5–7). Additionally, for first time, specific Sh proteins of 40–45 kDa most probably corresponding to some of the so-called short Sh cDNAs previously isolated by others have been identified. Using genetic criteria, it has been determined that at least a good part of this variety of proteins is generated by alternative splicing. Developmental experiments show a double wave of ShKch channel expression with a first peak at the third instar larval stage, a minimum at the beginning of pupation, and the highest plateau 36 h after hatching of adult flies. The pattern of Sh splice variants changes dramatically throughout development. A detergent-resistant fraction with about 50% of ShKch which seems to be anchored to submembranous properties has been found. Finally, other biochemical properties of ShKch, like membrane fractionation and glycosylation, are also described.

Potassium channels are the molecules mostly responsible for stabilizing the membrane potential of excitable cells thereby regulating their excitability (1). Most cells express various potassium channels with diverse functional and pharmacological properties (2). Thus, in the nervous system different types of potassium channels set the resting potential, control the duration and frequency of action potentials, determine the length of bursting periods, etc. In contrast to other ionic channels, this diversity and ubiquity, together with their small quantity, and the lack of good molecular markers have precluded the productive standard purification of most voltage-gated potassium channel proteins. Thus, the first cloning of a potassium channel gene was undertaken by means of a genetic approach (3–5). Homology cloning in different species and tissues together with site-directed mutagenesis and in vitro expression and in vivo have unravelled the existence of various voltage-gated potassium channel gene subfamilies and yielded a great increase in the knowledge of these molecules (see Refs. 6 and 7 for recent reviews). In spite of this great advance in molecular studies and a few brave purifications (8–11), the above mentioned difficulties have prevented successful investigation into the biochemical properties, tissular and cellular distribution, and other relevant data regarding the potassium channels proteins.

The Shaker locus of Drosophila harbors a transcription unit that by means of alternative and differential splicing at its 3’ and 5’ ends can produce a wide variety of transcripts encoding homologous proteins. The resulting channel isoforms share a common central core region and have different N-terminals and C-terminals (12–14) which confer differential electrical properties when expressed in Xenopus oocytes (15, 16). It has been shown that four Sh1 subunits assemble to form a functional potassium channel (17). Since properties of heteromultimeric ShKch differ from those of homomultimeric channels (18), the possible combination of Sh1 isoforms can provide in this way a molecular basis for potassium channel diversity in this organism.

Although, there have been previous reports on the preparation of antisera against Shaker proteins from Drosophila (19, 20), the theoretically expected variety of ShKch isoforms was not detected and the biochemical study of these proteins was not followed up.

We show here that by using immunochemical techniques the biochemical characterization of native Shaker potassium channels from Drosophila can be successfully approached.

EXPERIMENTAL PROCEDURES

Subcloning of cDNAs and Recombinant Protein Expression—Standard molecular biology techniques were performed as described (21). cDNA Shax and Shγ were kindly provided by O. Pongs. The fusion protein ShHX was produced by subcloning a HindIII-Xhol fragment of cDNA Shγ (nucleotides 205–828, Ref. 14) in the expression vector pMALcRI (New England Biolabs). The fusion protein was expressed and isolated as suggested by the supplier. The fusion protein ShSCX was produced by subcloning a BamHI-KpnI fragment (nucleotides 141–1185) of the same cDNA in the vector pE83 and expressing it as suggested (22). A PstI-BamHI fragment (nucleotides 100–373) of the cDNA Shw was cloned in the vector pE81 (22) in order to generate the fusion protein ShAPBX.

Antisera Production and Purification—Rabbit antisera against fusion proteins ShHX, ShSCX, and ShAPBX were produced with standard procedures (23). Nonspecific recognition of bacterial proteins was eliminated from all antisera by preabsorbing to total Escherichia coli proteins. Affinity purification was carried out using nitrocellulose matrices prepared with the corresponding purified recombinant fusion proteins. Mono-specific antisera thus obtained were tested for their specificity by mono-dimensional Western blot assays with total proteins from CS flies. Df flies which lack the genomic DNA from the Shaker coding region (13) were used as a negative control. In a second step, the most
specific antiserum were selected according to their affinity and titration by testing dilutions of the sera against decreasing amounts of the purified recombinant protein.

Flies Stocks, Culture, and Harvesting—Drosophila melanogaster stocks were grown on standard medium at 25°C with 12 h dark/light cycles. The Canton-S strain was used as a wild-type control although no differences were found with other common wild-type strains such as Berlin. Males of the mutant strains B55, WS2, C1, M3/0 and TX(2;X) labeled with white-X/Y were used (24).

Electrophoresis, Western Blotting, and Quantitative Immunoblots—In the course of this work three different electrophoretic techniques have been used: A, regular mono-dimensional SDS-PAGE was according to Laemmli (25). The concentration of acrylamide was 8% but the ratio acryl/bisacrylamide was lowered to 30/0.6 in order to increase the resolution of Sh proteins. Different tissue samples (whole flies, head, larval central nervous system, etc.) were homogenized at 80°C in 50 mM Tris, 25 mM KCl, 2 mM EDTA, 0.3 M sucrose, and 2% SDS (pH 7.4) with a microglass potter. Afterward, samples were boiled for 5 min in SDS-PAGE sample buffer and 20 μg of the total protein was loaded onto the gel. B, for some specific purposes, mono-dimensional gel electrophoresis modified from the method of Kyte and Rodriguez (26) was employed. Separation gels containing 7% acrylamide/bisacrylamide were used. C, bi-dimensional electrophoresis was carried out according to O'Farrell (27) with some modifications introduced by Santarén and Bravo (28). A 5–7 range of pH for the first dimension and 8% acrylamide gels for the second dimension was used.

Western blot was done by electrotransferring the proteins of the gel to nitrocellulose membranes under standard conditions. Immunoblots were carried out with monospecific anti-Sh antisera and a secondary goat anti-rabbit IgG antiserum coupled to horseradish peroxidase. Finally, the chemiluminescent ECL method (Amersham) for the detection of antibody binding was used. When required, laser densitometry and the computer program Image-Quant (Molecular Dynamics) were used for the quantification of immunoblots. The optical density obtained in this way was standardized against the total protein applied to the gel which was calculated by Coomassie Blue staining and densitometry of a gel run in parallel. All quantitative experiments were done at least in duplicate and repeated with two different sets of samples. Results are shown as average.

Preparation of Membranes and Fractionation—We have basically followed the method of Breer and Kniper (29) for the isolation of Drosophila neuronal membranes. Depending on the mass required, adult fly heads were homogenized either by vortexing anaesthetized flies on liquid nitrogen and sieving or cutting by hand with a scalpel. Heads were homogenized in manitol buffer (0.6 M manitol, 2 mM EDTA, pH 7.4) containing protease inhibitors (aprotinin, pepstatine, leupeptin, and E64 at 2 μg/ml and phenylmethylsulfonyl fluoride at 10 μM) and nucleases (DNase I and RNase A at 10 μg/ml). Membrane (P3) and cytosolic (S3) fractions were collected and P3 crude membrane were resuspended with 2% Ficol in manitol buffer, loaded on a discontinuous density gradient. We have found that by using 8, 12, 16, and 20% Ficol steps in manitol buffer instead of 5, 12, and 20% as described previously (29) and increasing the centrifuging conditions (126,000 × g for 90 min) we were able to further resolve in three different density fractions the previously reported single neuronal membrane fraction (29). The resulting membrane fractions were collected from the Ficoll interfaces and pelleted at 356,000 × g for 30 min. These pellets were analyzed by electron microscopy (EM) as described previously (29).

Solubilization of Membranes and Deglycosylation—Solubilization experiments were done by resuspending membrane pellets in 10 mM sodium phosphate buffer, 10 mM EDTA (pH 7.5) and stepwise addition of the appropriate detergent from a concentrated solution made in the same buffer. Unsolubilized material was pelleted by centrifuging 1 h at 48,000 × g. For deglycosylation assays, solubilized membrane proteins obtained with 1% Triton X-100 were diluted 1-fold with the buffer suggested by the enzyme supplier (Boehringer Mannheim), and digested with either mixed endoglycosidase F/N-glycosidase F (13 units/ml) or endo-α-N-acetylgalactosaminidase (O-glycosidase, 10 milliunits/ml), for 2 h at 37°C in the presence of protein inhibitors. Neuraminidase digestion was carried out in the same conditions but in 50 mM sodium acetate buffer (pH 5.4) with 0.7 unit/ml enzyme.

RESULTS

We have raised affinity purified polyclonal antiserum against various recombinant proteins obtained by expression of different cDNA fragments of the central core region common to all Sh transcripts. All of them recognized several specific Shaker proteins but after a selection procedure (see “Experimental Procedures”), one of them, called α-αShH1X1, showed the highest titer and affinity and, therefore, was used in all the experiments described in this paper.

An analysis by Western blot of total proteins from adult flies with α-αShX1 detects multiple bands with apparent molecular mass in the range of 65–85 kDa (Fig. 1A). The molecular size of the detected peptides is slightly larger than the theoretical molecular mass (65.1–76.2 kDa). All the labeled proteins must be specific to Shaker since the antiserum is monospecific and, moreover, these bands are absent in the track of control Df flies. Almost all the protein detected with the antisem is in the P3 membrane fraction. None of the bands seemed to appear in significant amounts in the cytosolic S3 extract (Fig. 1B), probably indicating the low occurrence or total absence of any intracellular pool of channels as that described for sodium and calcium channels (30, 31). The results presented here are in apparent contradiction to the large intracellular pool found in the case of Shaker-baculovirus infected insect cells (32) although, as the authors pointed out, this might be a consequence of hyperexpression. High resolution bi-dimensional electrophoresis and Western blot dramatically improves the resolution of immunolabeled bands. Thus, a homogenate of head proteins yields more than 10 spots with different pl and molecular mass in the range of 65–85 kDa (Fig. 1C). Again, all the immunostained spots seem to be specific since none of them is detected in the Df flies blot (Fig. 1D). Very interestingly, a
single spot of about 45 kDa at the middle of the pl range is also specifically labeled (Fig. 1C, arrowhead). Due to various reasons it is very unlikely that the observed multiplicity of bands could be generated by degradation of channel proteins. First, the denaturing conditions used in the preparation of samples (see “Experimental Procedures”) strongly preclude this possibility. Second, when a homogenate of head proteins prepared for bi-dimensional electrophoresis was left at room temperature for up to 5 h prior to being applied to the gel, no significant changes in the relative intensity of spots were observed (not shown), clearly indicating that the various proteins detected are not the result of proteolysis during sample preparation. Due to its size, the 45-kDa protein might correspond to one of the so-called short Shaker cDNAs isolated in a few laboratories (3, 12, 13). We have checked whether this short Shaker protein is in fact inserted in the plasma membrane. To this end, a protein extract of a membrane preparation from adult head (Fig. 1E) was analyzed. Except for some changes in the most acidic proteins, probably due to modifications in the phosphorylation state of the channel proteins commonly produced during the isolation of the membranes, the pattern of membrane proteins is very similar to that of total proteins. This experiment shows that the 45-kDa peptide is indeed in the plasma membrane.

In order to check whether this variety of protein detected by our antiserum is in fact generated by alternative splicing at the Shaker locus, a genetic approach has been taken. Previously, by using various recombinant Sh proteins containing the central common core region and different alternative amino and carboxyl ends, it has been found that antisem α-ShH1X1 is able to equally recognize all Sh Kch isoforms (not shown). The chromosomal break point W32 interrupts the Shaker transcription unit between exon 2 and 3 (13) but does not completely preclude the transcription (14), probably through the use of a second promoter located downstream of the translocation point. Therefore, at least exons 1 and 2 cannot be transcribed and the corresponding alternative splice transcripts ShB and ShD (in the nomenclature of Ref. 13) are never produced in this mutant. As expected, the bi-dimensional immunoblot pattern of W32 flies lacks a few of the peptides labeled in wt flies (Fig. 1, C and F) clearly indicating that the complex pattern of Sh proteins is indeed generated by alternative splicing. The fact that short Sh proteins also disappear in W32 flies, gives clear evidence for its specificity and additionally indicates that the 45-kDa peptide must belong to either ShB or ShD N-terminal isoforms. A couple of very close spots running at equal molecular size on the most acidic side of the gel are not detected in this mutant (Fig. 1C, arrowhead), whereas another protein on the most basic side of the W32 map increases its intensity in respect to wt (Fig. 1F, arrowhead). Thus, the question remains as to why this smaller 45-kDa Sh peptide could not be detected by mono-dimensional SDS-PAGE and Western blot (as in Fig. 1A, for instance). One explanation could be the differences in the manner of preparing samples for each type of electrophoresis. Accordingly, a modified mono-dimensional electrophoretic method which uses concentrated urea instead of SDS as denaturating agent (26) was used. Under these experimental conditions an intense band of 45 kDa and a weaker one of 41 kDa together with a few intense bands in the range of 65–90 kDa were specifically labeled (Fig. 1G). Moreover, when an urea/Nonidet P-40 protein extract as that used for bi-dimensional electrophoresis of Sh head proteins (Fig. 1C) was heated in the presence of SDS and run in a regular SDS-PAGE gel, peptides in the range of 65–85 kDa were normally labeled while the 45-kDa protein was no longer detected (Fig. 1H). Altogether, these results point to a probable aggregation and precipitation of the short Sh peptides during boiling with SDS. This can also explain why these truncated channel proteins have not been detected in previous work by other laboratories (19, 20).

A quantitative immunoblot analysis of the anatomical distribution of Sh Kch has been carried out. As shown in Fig. 2A, Sh proteins are more abundant in the head than in thorax, and they are practically absent in the abdomen of adult flies. This distribution of Sh Kch proteins correlates well with that of nervous tissue. Additionally, with this simple anatomical section one can observe that the pattern of Sh proteins of head and thorax are rather different (Fig. 2C), clearly indicating a tissue-specific alternative splicing as previously suggested by Northern blot analysis (14).

Protein extracts of developmentally staged samples of Dro sophila have been analyzed by quantitative immunoblotting with the antisem α-ShH1X1. As shown in Fig. 3A, there are two waves of Sh Kch expression during development. Sh proteins are first clearly detected in first instar larvae and increase in intensity up to the early third instar. At the beginning of puperation they seem to disappear. The expression restarts at the late pupal stage and continues to increase up to the adult fly. Changes in the pattern of bands can be nicely viewed in bi-dimensional gels. Thus, protein extracts from larval brain (Fig. 3B) were prepared and their bi-dimensional immunoblot was compared to that of adult head (Fig. 1C) since both structures hold more than 90% of the Sh proteins present in their respective developmental stages. The pattern of adult brain is clearly more complex than that of larval central nervous system. This lacks most of the high molecular size proteins and shows no more than four labeled spots that seem to have their counterpart in the adult bi-dimensional map including a weak labeling of the 45-kDa spot. As stated above, immunoblots carried out from mono-dimensional gels under normal conditions did not detect a significant amount of Sh channels in protein extracts from embryo (Fig. 3A). In situ hybridization with a variety of probes has also failed in detecting Sh transcripts in embryos (33). However, Sh potassium channel currents have been recorded in embryonic cells (34). Therefore, we have tried to improve the detection level by overloading bi-dimensional gels and increasing the time of autoradiography in the final chemiluminescent reaction of the immunoblot. Under these experimental conditions Sh proteins could finally be detected (Fig. 3C) indicating that even at considerably lower comparable levels Sh Kch are indeed expressed in embryonic
P-40, and Triton X-100, were efficient enough in solubilizing detergents tested, including CHAPS, deoxycholate, Nonidet. Membrane fractions were solubilized. Most common biological out.

samples. However, with this kind of analysis the possibility that Sh isoforms with a low level of expression might be segregated in different kinds of membranes cannot be ruled out. In order to further biochemically study the Sh Kch proteins, membrane fractions were solubilized. Most common biological detergents tested, including CHAPS, deoxycholate, Nonidet, and Triton X-100, were efficient enough in solubilizing these channel proteins. As can be seen in Fig. 5A, increasing concentrations of detergents progressively solubilized Sh protein, reaching a plateau at around 50% of the channel proteins. Addition of either the same or other detergents to the insoluble fraction did not solubilize further significant amounts (Fig. 5B). This has lead to the conclusion that the detergent-resistant fraction of Sh Kch is the same for all non-denaturating detergents. In order for the solubilization to be completed, strong denaturating agents such as urea (Fig. 5B) or boiling in SDS had to be used. It must be also mentioned here that both solubilized and insoluble fractions have a very similar pattern of bands regardless of the detergent and concentration used (Fig. 5C), probably indicating that at least the most abundant Sh Kch isoforms are present with similar ratios in both fractions.

Other well known voltage-sensitive channels such as sodium channels have shown a wide variety of post-translational modifications (35). Here the biochemical characterization of post-
Fig. 5. Solubilization of Shaker potassium channel proteins. A, total membranes of Drosophila head were solubilized with increasing concentrations of either deoxycholate or Triton X-100. Solubilized and insoluble fractions were analyzed by electrophoresis and Western blot and the relative amounts of Sh proteins determined by densitometry as explained elsewhere. B, sequential solubilization of membranes with two detergents was also carried out as schematically shown. Concentrations of detergents and other compounds are as indicated. The rest of experimental conditions are as those used for solubilization in a single step. C, detail at high magnification of a mono-dimensional immunoblot containing soluble and insoluble fractions generated with 0.3% deoxycholate.

translational modifications of Sh Kch has been initiated by studying its glycosylation. Because of probable interference of deoxycholate with some glycosylases, Triton X-100 soluble fractions were used for this purpose. As shown in Fig. 6, N-glyopeptidase F produces some sharpening and a clear shift of about 6 kDa to lower M, in all bands without any apparent change in the relative positions of the major bands. Other common glycosidase such as neuraminidase and O-glycosidase did not produce any apparent change in the electrophoretic mobility of Sh proteins (not shown), suggesting that they are only N-glycosidated and that, in contrast to rat brain potassium channels (36), Sh Kch of Drosophila lack sialic acid in their carbohydrate chains.

DISCUSSION

Multiple potassium channel isoforms can presumably be generated by splicing at the Shaker locus of Drosophila (12-15). This has raised the question of whether this theoretical repertoire of Sh Kch variants is in fact expressed and how it is used. With our immunochemical approach we have detected a high number of proteins in different anatomical and developmental preparations. The genetic control with Df flies makes the specificity of the detected proteins undoubtable, whereas the control with the mutant W32 demonstrates that the multiplicity of detected Sh peptides is at least in part generated by alternative splicing although post-translational modifications of Sh Kch may also contribute to the complexity of the electrophoretic pattern. Our results clearly contrast with work previously reported by other laboratories which, in spite of the diversity of cDNAs isolated, have shown single bands in Western blot analysis (19, 20). This is most probably due to the increase in resolution of the electrophoretic techniques used and/or to a higher affinity of the antisera. As we have shown in this paper, the wide repertoire of Sh Kch isoforms in Drosophila is differentially expressed both as tissue as well as in a developmentally regulated manner. Previous reports using in situ hybridization (33), polymerase chain reaction (37), and reporter gene DNA engineering (38) have indicated that Shaker gene transcripts are differentially expressed in some Drosophila tissues. In other organisms where neuronal electrical recording is easier, sharp changes in the electrical properties of neurons take place throughout development together with abrupt changes in the densities and kinetics of potassium currents (see Ref. 39 for a recent review). In the same way, two Shaker potassium type A currents with different electrical properties were recorded during the differentiation of Drosophila myotubes showing a progressive replacement of an early type A current by a late mature one (34). Therefore, our results demonstrating changes in the expression of Sh Kch isoforms during development probably reflect modifications in potassium conductance and, as a result, in the neuronal electrical properties. Further work with isoform specific antibodies will be necessary to identify the Sh Kch variants expressed in different tissues at developmentally defined stages.

Together with the regular full-length Sh transcripts previously described, the isolation of short Sh cDNAs that would theoretically produce truncated channel subunits has been reported by a few laboratories (3, 12, 13). The fact that the corresponding short proteins were not detected in previous studies (19, 20) has made the actual existence of these truncated proteins doubtful until now. For the first time some specific short Sh proteins have been detected in this work. They most probably correspond to the so-called short Sh mRNAs under various criteria. First, their size (40-45 kDa) fit nicely with that deduced from the cDNAs having the first three (S1-S3) out of the six membrane expanding domains of a potassium channel subunit (3, 13) and the possibility of being glycosylated at the S1-S2 loop (5, 12, 13). Second, they are detected by our antisera a-SHXX1 which has been raised against a recombinant peptide which contains a cytoplasmic stretch common to all Sh isoforms, the S1 segment and the whole S1-S2 loop. Third, most short Sh peptides are eliminated by the mutation W32. Thus, our results are the first evidence for the existence of native truncated Sh proteins and raise the question of their biological role. Although it has here been demonstrated that the 45-kDa peptide reaches the plasma membrane, it is very unlikely that such a truncated Sh protein could drive a potassium current by itself since it lacks three (S4-S6) out of the six membrane expanding domains of a voltage-gated potassium channel subunit and consequently it is missing some of the most important structural features of a functional channel (6). However, the short Sh protein does contain the complete N-terminal and S1 domain where the structural elements mostly responsible for the assembly of channel subunits have been localized (40, 41). Accordingly, recombinantly produced short potassium channels insert in the membrane and seem to form heterotetramers with regular large subunits (42). Coinjection in Xenopus oocytes of mRNAs corresponding to large and truncated Sh Kch produce a decrease in the expressed potassium current (41) suggesting a dominant-negative role. Transgenic flies carrying a heat inducible gene encoding a truncated Sh Kch together with regular doses of the wt gene show a Shaker phenotype after heat shock (43). Interestingly, a truncated splice variant of the mouse Kv1-5 potassium channel also shows a dominant-negative effect (44). A possible dominant-
negative function of the truncated Sh Kch subunits is a very attractive hypothesis which needs to be further explored. Our experiments have shown that the major Sh Kch proteins are evenly distributed in neuronal membranes of different composition and, therefore, strongly suggest a lack of subcellular segregation of major Sh isoforms. Conversely, as mentioned previously, there are reports indicating that certain Sh isoforms are differentially expressed in some areas of the nervous tissue and retina (33, 37, 38). Nevertheless, our results are not in contradiction with those since extensive work on separation of nervous tissue membranes has demonstrated that fractionation by density centrifugation takes place more as a function of the subcellular nature of membranes than as a result of their cellular origin (29, 45). In principle, an alternative explanation to our results can be that similar Sh Kch isoforms are detected in membranes at various steps of biosynthesis. However, this is very unlikely since immature channel proteins show a distinct electrophoretic mobility due to differential glycosylation (35, 46). The solubilization experiments carried out suggest that about half of the Sh Kch of adult fly brain remains anchored to some submembranous structures and in this way they are resistant to detergents. Other authors have found a similar percentage of solubilized channels in Shaker transfected cells (47). Interestingly, our detergent resistant pool has the same pattern of Sh isoforms than the solubilized one. This suggests that the structural elements responsible for such an interaction must be within the region common to all Sh Kch subunits. Membrane-cytoskeleton interactions as those found for other ion channels and that seem to play an important role in their distribution and clustering (48, 49) appear as the most likely candidate.

Native Sh Kch proteins from Drosophila have been here demonstrated to be N-glycosylated. The equal shift to lower size of Sh proteins after complete enzymatic deglycosylation leads to the conclusion that at least the major Sh Kch isoforms are glycosylated to a similar extent and probably at the same site(s). However, the contribution of the carbohydrate chains to Sh Kch in different cellular systems (32, 46, 47) range from 16 to 44 kDa which is much greater than the 6 kDa that we have found in native channels. Two consensus sites for N-glycosylation can be found in the sequence of Shaker transcripts at the S1-S2 loop in a region common to all splice variants (5, 12, 13) and both are modified in Shaker channels expressed in Xenopus oocytes (32) and mammalian cells (46). Unfortunately, with the present data we cannot determine whether one or both sites are glycosylated in the native Sh Kch.

We trust that the immunochemical approach used here can be generally applied to other channel molecules which have already been cloned and present serious difficulties in their standard purification.

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